Published in final edited form as:

J Trace Elem Med Biol. 2014 July ; 28(3): 311–316. doi:10.1016/j.jtemb.2014.02.002.

Differential metal content and gene expression in rat left ventricular hypertrophy due to hypertension and hyperactivity

Meenakumari Subramanian¹, Adam L. Hunt², Giuseppe A. Petrucci², Zengyi Chen³, Edith D. Hendley¹, and Bradley M. Palmer¹

¹Department of Molecular Physiology and Biophysics, University of Vermont, Burlington, VT 05405

²Department of Chemistry, University of Vermont, Burlington, VT 05405

³Department of Medicine, University of Vermont, Burlington, VT 05405

Abstract

The spontaneously hypertensive rat (SHR) has been studied extensively as a model of left ventricular hypertrophy (LVH) and associated cardiac dysfunction due to hypertension (HT). The SHR also possesses a hyperactive trait (HA). Crossbreeding SHR with Wistar-Kyoto (WKY) control rats, which are nonHT and nonHA, followed by selected inbreeding produced two additional homozygous strains: WKHT and WKHA, in which the traits of HT and HA, respectively, are expressed separately. WKHT, WKHA and SHR all display LVH, but only the SHR exhibits cardiac dysfunction. We hypothesized that cardiac dysfunction in the SHR is uniquely characterized by calcium overload. We measured total cardiac Ca, Cu, Fe, K, Mg and Zn in the four strains. We found elevated Ca and depressed Cu, Mg and Zn with HT, but not unique to SHR. We surmise that HT promotes aberrant regulation of cardiac Ca^{2+} , Cu^{2+} , Mg^{2+} and Zn^{2+} , which does not necessarily result in cardiac dysfunction. Interestingly, Cu was elevated in HA strains compared to nonHA counterparts. We then analyzed gene expression as mRNA of Cu-containing proteins, most notably mitochondrial-Cox, Dbh, Lox, Loxl1, Loxl2, Sod1 and Tyr. The gene expression profiles of Lox, Loxl1, Loxl2 and Sod1 were found especially high in the WKHA, which if reflective of protein content could account for the high Cu content in the WKHA. The mRNA of other genes, notably Mb, Fxyd1, Maoa and Maob were also examined. We found that Maoa gene expression and monoamine oxidase-A (MAO-A) protein content were low in the SHR compared to the other strains. The finding that MAO-A protein is low in the SHR and normal in the WKHT and WKHA strains is most consistent with the idea that MAO-A protects against the development of cardiac dysfunction in LVH but not against LVH in these rats.

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Address for Correspondence: Bradley M. Palmer, Ph.D., 122 HSRF Beaumont Ave., Department of Molecular Physiology and Biophysics, University of Vermont, Burlington, VT 05405, bmpalmer@uvm.edu, phone: (802) 656-2650, fax: (802) 656-0747.

Conflict of Interest Statement

As corresponding author, I assure the editors of the Journal of Trace Elements in Medicine and Biology that none of the authors have a conflicting interest with regards to the results or publication of the submitted article.

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Keywords

cardiac hypertrophy; calcium; zinc; copper; monoamine oxidase

INTRODUCTION

The spontaneously hypertensive rat (SHR) is characterized by hypertension (HT) and hyper-reactivity to stress associated with hyperactivity (HA). While the SHR has been used extensively as a model of left ventricular hypertrophy (LVH) and associated dysfunction due to severe HT, the cause of LVH in this strain is not solely due to HT [1–4]. The HT and HA traits of the SHR were segregated into two new homozygous strains: the WKHT, which is HT and not hyperactive (nonHA), and the WKHA, which is HA and not hypertensive (nonHT) [5, 6]. By examining four genetically related strains it was possible to look for correlations of biological findings with the HT trait, if present in SHR and WKHT but not in WKY and WKHA, or with the HA trait, if present in SHR and WKHA but not in WKY and WKHT.

Both the WKHT and WKHA strains develop LVH by mechanisms independent of HA and HT, respectively [5, 7]. Yet, neither the WKHT nor WKHA strain develops cardiac dysfunction at a cellular level like that observed in the SHR [8]. In the current study we used these four strains to test hypotheses regarding the role of metal regulation, particularly calcium overload, in the development of LVH and cardiac dysfunction in the SHR.

It has been suggested that LVH and associated dysfunction like that in the SHR are attributable to suppressed function of calcium regulatory proteins that remove Ca^{2+} from cardiomyocytes [8, 9]. Excess calcium load may also initiate gene expression leading to LVH [10, 11]. We have recently reported that Zn^{2+} can displace intracellular Ca^{2+} in isolated cardiomyocytes [12]. We therefore hypothesized that cellular calcium overload and/or zinc deficiency, measurable as total cardiac content of Ca and Zn, are present in the SHR. We found elevated Ca and depressed Cu, Mg and Zn with HT, but not unique to SHR. We also found elevated Cu with HA. Further examination of gene expression, especially for copper-proteins, revealed an elevated gene expression of the family of lysyl oxidase enzymes and superoxide dismutase in the WKHA. The gene expression and protein content of monoamine oxidase-A was found especially only reduced in the SHR, which suggests protection against development of cardiac dysfunction with LVH in the other strains.

METHODS

Rat models

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Vermont College of Medicine and complied with the *Guide for the Use and Care of Laboratory Animals* published by the National Institutes of Health. Male rats of four inbred (fully homozygous) strains were used in this study. SHR and WKY were obtained commercially from Harlan Sprague-Dawley (Indianapolis, IN). Two other inbred strains were derived previously from crossing the SHR and WKY: WKHA,

hyperactive, and WKHT, hypertensive rats [6]. WKHA (F45-F46 generations) and WKHT (F42-F45 generations) were attained from the colony maintained at the University of Vermont since 1980 and presently available from the Rat Resource and Research Center, Columbia, MO. At 10 weeks of age, four male rats from each strain were anesthetized with isoflurane, and hearts were removed, flash frozen in liquid nitrogen and stored at -80°C .

Cardiac Elemental Analysis

Polypropylene bottles were soaked in 0.4 mM EGTA overnight, then rinsed four times with ddH₂O and allowed to drip dry according to published recommendations [13]. Between 40–80 mg of each LV sample was placed in its own tube and lyophilized by 90 min of vacuum freeze drying. Samples were digested in 10 μL nitric acid (15.8 Normal) per mg tissue wet weight. ddH₂O was added to bring total volume to 1000 $\times$ dilution of original tissue wet weight including final concentrations of 0.8 N nitric acid. Standards were prepared for elements Ca, Cu, Fe, K, Mg and Zn including equivalent concentration of nitric acid. Detection of elemental content was measured in duplicate using inductively coupled plasma atomic emission spectroscopy (ICP-AES) performed by the ULTIMA2C operated in mono-mode (Horiba Scientific, Edison, NJ).

mRNA analysis and Quantitative PCR

Total RNA was extracted from the rat hearts using Qiagen RNeasy kit and cDNA was synthesized using Invitrogen Superscript III first-strand synthesis system according to manufacturer instructions. Sequences for the Taqman specific primers were obtained from Applied Biosystems (<http://www.appliedbiosystems.com>). mRNA expression levels were measured in duplicate using a ABI Prism 7900HT sequence detection system based by polymerase chain reactions (PCR). C_t was calculated relative to average C_t value for HPRT expression. The average C_t for all WKY samples was used as the normalization value to calculate C_t . C_t was then calculated for relative fold difference in transcription levels, and relative quantification (RQ) values calculated as 2^{-C_t} .

Single nucleotide polymorphisms (SNP) rs8148546 and rs13458141 for Maa and rs64072569 for Loxl2 were tested in cardiac cDNA of all rat strains by PCR. The PCR conditions included denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 72°C for 30 s and extension at 72°C for 30 s with the final extension of 72°C for 10 min. Restriction pattern was determined on a 2% agarose gel using restriction digestion enzymes specific for the polymorphism. These SNPs were not detected and therefore did not influence the gene expression levels reported here.

Analysis

All analyses were performed using SPSS v. 19 (SPSS Inc.). Values reported in tables and figures as mean $\pm$ sem. Rats were identified for hypertensive trait as HT or nonHT and activity trait as HA or nonHA. We applied a $2(\text{HT, nonHT}) \times 2(\text{HA, nonHA})$ repeated-measures ANOVA to all variables to differentiate effects on elemental content and gene expression. Post-hoc analyses were applied among groups using Student-Newman-Keul's post-hoc test. Statistical significance is reported at $P < 0.05$ level and also at $P < 0.10$ to show strong trends which are in agreement with statistically significant results and thus to

minimize the possibility of committing a Type II (false-negative) error [14]. Specifically, we note a $P < 0.10$ statistic if it is in support of other conventionally significant findings at the $P < 0.05$ level, such as when ANOVA results are conventionally significant, and the post-hoc analyses show trends in support of the ANOVA results.

RESULTS

Rat Characteristics

Characteristics of the four homozygous rat strains measured over a ten year period including activity, blood pressure, heart rate and body mass are presented in Table 1 as previously reported [8]. The WKY strain is considered the normal control and thus displays normal blood pressure and activity. Activity is detected by movements recorded in an activity cage. WKHT rats are severely hypertensive and display blood pressures similar to those of the SHR. While the WKHT rats are somewhat more active than the WKY, the WKHT rats are clearly not hyperactive on the order of the WKHA and SHR and do not display elevated catecholamine release with stress as observed in the WKHA and SHR [15]. The WKHA rats are hyperactive on the order of the SHR, but are clearly not hypertensive on the order of the WKHT and SHR.

Other characteristics of the four strains of particular relevance to cardiac function include the following: 1) WKHT, WKHA and SHR all display LVH compared to WKY controls (Figure 1A); 2) hypertension in the SHR and WKHT is associated with hypertrophy of stellate ganglion cells that innervate the heart [16]; and 3) HA in the WKHA and SHR rats is associated with a hyper-reactivity to stress and elevated catecholamine release during stress [15].

Cardiac Elemental Analysis

We had expected to find elevated Ca content in the SHR compared to the other strains. We found Ca content elevated by ~25% with HT as determined by ANOVA, but not unique to the SHR strain (Table 2 and Figure 1B). In other words, the significant HT main effect refers to Ca in the WKHT and SHR being statistically higher than that in the normotensive WKY and WKHA control strains, respectively, and there were no unique post-hoc findings to highlight a significant difference between any two groups. We also found a significant reduction in cardiac content of Cu by ~25%, Mg by ~10% and Zn by ~13% in HT strains compared to their nonHT controls. There was also a tendency ($P = 0.127$) for HT associated reduction in K by ~10%, but no statistical significance. There were no significant effects of HT or HA on cardiac content of Fe.

Surprisingly, there was a significant elevation in Cu with HA. As illustrated in Figure 2A, cardiac Cu content was reduced in HT compared to nonHT controls and elevated in HA compared to nonHA counterparts. These findings led us to examine whether specific Cu-containing proteins may be responsible for cardiac Cu content observed in these strains.

Copper-containing proteins

Those Cu containing proteins that might be expected in cardiac muscle are most notably cytochrome c oxidase, dopamine β -hydroxylase, the family of lysyl oxidases, and superoxide dismutase.

Mitochondrial cytochrome c oxidase contains 3 Cu atoms and is made up of 14 subunits [17]. We examined the expression of the gene for the subunit Cox3 as a proxy indicator of cytochrome c oxidase expression. We found high Cox3 gene expression in the three rat strains with LVH (Table 3 and Figure 2B).

Dopamine β -hydroxylase (gene Dbh) converts dopamine to norepinephrine in the sympathetic nervous system, including the stellate ganglion that innervates the heart. We did not detect any cardiac Dbh expression in any of these rat strains.

Lysyl oxidase (gene Lox) and lysyl oxidase-like proteins (genes LoxlX) are a family of Cu containing enzymes that deaminate lysine side chains that form cross-links in fibrillar collagen and elastin. Our studies revealed detectable levels of mRNA levels of Lox, Loxl1 and Loxl2 that varied among the strains. The gene expression of the Loxl1 and Loxl2 display a pattern among the strains visually similar to that of Cu (Figure 2C).

Superoxide dismutase (gene Sod1) converts superoxide ($O_2^{\bullet-}$) to O_2 and H_2O_2 and plays an important role as an antioxidant in cardiac tissue [18]. The enzyme requires one Cu atom and one Zn atom as cofactors. We detected both HT and HA main effects for Sod1 expression as well as an HT $\times$ HA interaction by ANOVA. These statistics result from the especially high expression in the WKHA compared to the other three strains, whose mRNA levels were within 15% of each other (Figure 2D).

Tyrosinase (gene Tyr) is a copper enzyme that catalyzes the production of melanin from tyrosine. We found effectively no expression of Tyr in the hearts of these rat strains.

Other proteins of possible interest

Myoglobin (gene Mb) is an Fe-binding heme molecule found in muscle tissues. We found high expression of Mb in all strains, but not differentially expressed.

Phospholemman (gene Fxyd1) is a sarcolemmal protein that modulates Na^+ - K^+ ATPase and Na^+ - Ca^{2+} exchanger in cardiomyocytes [19]. Gene expression of this Fxyd1 was not different among the strains examined here.

The enzyme MAO-A deaminates serotonin and the catecholamines epinephrine, norepinephrine and dopamine. Interestingly, the WKHT rats showed the highest levels of Maa gene expression among the strains examined here (Table 3), and the SHR showed the lowest levels. Protein levels of MAO-A enzyme were markedly reduced in the SHR compared to the other rat strains (Figure 3).

MAO-B deaminates dopamine. The WKHA expressed the highest level of Maob, which was significantly greater than that of the other three strains (Table 3). This finding is likely

related to the prominent role played by brain dopamine in behavioral hyperactivity disorder seen in humans as well in hyperactive rats [20].

DISCUSSION

The SHR rat has been especially useful in examining mechanisms of LVH-associated cardiac dysfunction. The development of LVH in the SHR is often thought to develop in response to severe systemic HT. However, LVH can develop in the SHR strain independent of HT [1–4]. The current study highlights the advantage of examining four genetically related strains in which HA and HT traits are inherited in all possible combinations. This combination allows us to search for biological changes more powerfully than if we had only SHR (HA and HT) vs. WKY controls to model these traits.

Cardiac metal regulation in LVH

Intracellular Ca^{2+} regulation is impaired in the SHR and to a large degree accounts for cardiac dysfunction in the SHR [9]. The other two rat models of LVH we examined here, WKHT and WKHA, do not display Ca^{2+} dysregulation or adverse cardiomyocyte contraction-relaxation function [8]. We had expected to find calcium overload in the hearts of SHR, but found instead calcium overload associated with HT in both the WKHT and SHR. We also found that HT leads to a reduced cardiac content of other metals, notably Cu, Mg and Zn. While we cannot offer explanations for the dysregulation of these metals associated with HT, these data justify further examinations into the plasma content vs. cellular content of these and other elements whose regulation is affected by HT. In particular, Zn^{2+} can inhibit inward Ca^{2+} current through the L-type calcium channel [12, 21]. We speculate that the elevated cardiac Ca content in the HT rats could reflect a lowered inhibition of inward Ca^{2+} current due to relatively low Zn^{2+} in HT.

We attempted here to discern causes for the lowered Cu with HT and elevated Cu with HA. The elevated Cu content in the HA strains bearing LVH raises the possibility that Cu can play a role in development of LVH. Cooper et al. [22] have demonstrated that LVH can be ameliorated after 6–12 months of Cu-chelation therapy in type-2 diabetic patients with LVH and normal ejection fraction. It is conceivable then that an elevated Cu or upregulated Cu-sensitivity mechanism within a yet-unidentified Cu pool may initiate LVH independent of HT such as in the WKHA and possibly in the SHR that has been rendered normotensive [2, 3].

One such Cu-dependent mechanism involves Cu-dependent phosphorylation of extracellular signal-related kinase (ERK). The high circulating catecholamine levels in the WKHA and SHR strains [15] would be expected to result in pronounced β -adrenergic stimulation, which in turn is expected to initiate LVH through phosphorylation of ERK1/2 [23]. Turski et al. [24] have demonstrated that Cu must bind to the MAPK/ERK kinase MEK1 prior to phosphorylating ERK1/2. Although this is speculative at this time, these reports implicate a Cu-dependent β -adrenergic/MEK/ERK pathway leading to LVH that is upregulated in the HA strains and independent of HT.

Furthermore, the WKHA with its higher expression of Sod1 is likely more protected from oxidative damage caused by superoxide. Superoxide is a highly toxic oxygen radical that can lead to atherosclerosis and the development of hypertension via enhanced systemic vascular resistance [18]. The enhanced expression of Sod1 in the WKHA would be expected to protect against inflammation as might arise from ischemia-reperfusion injury as well as protect against myocardial apoptosis and fibrosis [25]. For example, there is no overt fibrosis of the WKHA heart compared to the WKY despite clear LVH in the WKHA [26].

Monoamine oxidase-A and LVH

Hydrogen peroxide is generated by MAO-A as a byproduct of its deamination of serotonin and catecholamines [27]. It has been suggested that the MAO-A associated oxidative stress signals LVH after activation of ERK1/2 signaling pathways [28]. Another study in mice also suggests that MAO-A exacerbates development of LVH and cardiac dysfunction via elevated oxidative stress associated with catecholamine breakdown [29]. A study from Lairez et al. [30] however conversely reports an elevated risk of developing serotonin-dependent LVH and dysfunction after pressure overload in the MAO-A knockout mouse, thus suggesting MAO-A lends protection against serotonin-related development of LVH and dysfunction. We were curious if MAO-A expression in the SHR would lend support to either of these interpretations.

We found the expression of Maa gene and the content of MAO-A protein low in the SHR. We were surprised to find relatively normal to high expression of Maa in the WKHT and normal to high content of MAO-A protein in the WKHT and WKHA, which bear similar LVH but no cardiac dysfunction. One possible explanation for the elevated MAO-A protein levels in the WKHT and WKHA may be related to the selection process used in breeding the strains to homozygosity. The WKHT was selected for low activity in the context of high blood pressure. A normal or high level of MAO-A in the WKHT is consistent with reduced catecholamine production associated with lowered activity and reactivity to stress. The WKHA was selected for high activity and low blood pressure, thus the genome may have been fixed to include a normal to high level of MAO-A as a part of an anti-hypertensive strategy. In these cases, the MAO-A could not be the only factor leading to the separation of the HT and HA traits, and other factors must contribute to separation of the HT and HA traits in these strains. The low MAO-A content in the SHR, on the other hand, conceivably contributes to both HT and HA traits in this strain.

With regards to LVH development, our findings are most consistent with the idea that MAO-A does not protect against development of LVH in the context of HT, as both WKHT and SHR have similar levels of HT and LVH but possess different levels of MAO-A and Maa expression. Our findings rather suggest that MAO-A may play a role in protection against cardiac dysfunction in the context of LVH, as both WKHT and WKHA with LVH and normal to high MAO-A do not display cardiac dysfunction yet SHR with LVH and low MAO-A displays significant cardiac dysfunction.

Conclusion

Our use of the multiple rat strains examined here allowed us to test whether cardiac metal content was strictly associated with development of LVH and cardiac dysfunction in the SHR. With the inclusion of the WKHA we were able to conclude that the state of HT and not LVH is associated with aberrant cardiac content of Ca, Cu, Mg and Zn. We would further infer that dysregulation of these metals in HT cannot be the sole cause of cardiac dysfunction in the SHR. The elevated cardiac Cu associated with LVH in the HA strains raises the possibility of a Cu-sensitive pathway in LVH development as noted by others [22].

Acknowledgments

This work was supported by NIH grant R01 HL086902. We thank the DNA Analysis Facility at the Vermont Cancer Center for performing real-time quantitative PCR. We also thank Ms. Gabriela Mora for her expert technical assistance in the ICP-AES laboratory at the Department of Geology, University of Vermont. The ICP-AES instrument was provided by an NSF grant to Dr. Paul Bierman, EAR0132169.

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Figure 1.

LVH and Ca content. **A.** LV mass per body mass was significantly higher in the WKHT, WKHA and SHR compared to WKY. **B.** Cardiac Ca content was elevated in HT strains compared to nonHT as indicated by significant ($P < 0.05$) HT main effect by ANOVA. a=different from WKY, b=different from WKHT, c=different from WKHA, d=different from SHR at $P < 0.05$ level by S-N-K. n=4 in each group.

Figure 2.

Cardiac Cu content and gene expression of Cox3 and Lox-family genes. **A.** Total cardiac Cu content is reduced in the HT strains compared to nonHT strains, which is seen as the tendency for Cu in the SHR and WKHT to be lower than that in WKHA and WKY, respectively. Also, cardiac Cu is elevated in the HA compared to nonHA strains, which is seen as the tendency for Cu in the WKHA and SHR to be higher than that in WKY and WKHT, respectively. **B.** mRNA expression of Cox3 was elevated in all three strains displaying LVH irrespective of HT or HA. **C.** mRNA expression levels for Lox11 (black) and Lox12 (grey) were normalized to expression in the WKY. Expression of Lox11 and Lox12 qualitatively appear to reflect the Cu content among the strains. **D.** mRNA levels of Sod1 were especially high in the WKHA compared to the other three strains. a=different from WKY, b=different from WKHT, c=different from WKHA, d=different from SHR at $P < 0.05$ level by S-N-K. Underline indicates trend at $P < 0.10$ level consistent with $P < 0.05$ results. n=4 in each group.

Figure 3.

Western blot analysis of MAO-A protein content. **A.** MAO-A protein content appears lower in SHR compared to other strains. **B.** MAO-A content normalized to GAPDH was significantly lower in the SHR strain. a=different from WKY, b=different from WKHT, c=different from WKHA, d=different from SHR at $P<0.05$ level by S-N-K. Underline indicates trend at $P<0.10$ level consistent with $P<0.05$ results. $n=4$ in each group.

Table 1

Characteristics of inbred strains from the University of Vermont colony, 1996 to 2005.

Activity Group	Not Hyperactive		Hyperactive	
Blood Pressure Group	Not Hypertensive	Hypertensive	Not Hypertensive	Hypertensive
Males	WKY	WKHT	WKHA	SHR
Number of rats	54	80	93	39
Activity (counts/min)	257 ± 13 ^{bcd}	343 ± 15 ^{acd}	572 ± 17 ^{ab}	586 ± 22 ^{ab}
Mean pressure (mmHg)	100 ± 2 ^{bcd}	140 ± 2 ^{acd}	110 ± 1 ^{abd}	149 ± 2 ^{abc}
Diastolic pressure (mmHg)	88 ± 1 ^{bcd}	125 ± 2 ^{acd}	96 ± 1 ^{abd}	132 ± 2 ^{abc}
Systolic pressure (mmHg)	122 ± 2 ^{bcd}	170 ± 2 ^{acd}	138 ± 2 ^{abd}	183 ± 3 ^{abc}
Heart rate (bpm)	323 ± 5 ^{cd}	319 ± 4 ^{cd}	353 ± 3 ^{ab}	342 ± 5 ^{ab}
Body mass (g)	309 ± 3 ^{bcd}	318 ± 3 ^{acd}	292 ± 3 ^{ab}	297 ± 3 ^{ab}

^a = different from WKY,^b = different from WKHT,^c = different from WKHA,^d = different from SHR by S-N-K at $P < 0.05$ level.

Table 2

Cardiac elemental content measured by ICP-AES.

Element (ppm)	WKY	WKHT	WKHA	SHR	ANOVA
Ca	45.7±4.2	52.2±6.0	44.4±3.2	49.2±3.3	HT
Cu	2.36±0.31 ^c	1.64±0.44 ^{cd}	4.00±0.31 ^{abd}	2.88±0.22 ^{bc}	HT, HA
Fe	52.0±1.33	52.5±3.01	48.9±1.35	50.7±0.74	
K	3578±472	2955±97	3680±269	3366±283	
Mg	242±11	220±11	240±5	225±6	HT
Zn	19.6±0.7	18.4±0.5	20.2±0.8 ^d	17.5±0.5 ^c	HT

ppm=μg of element per g wet weight of L.V. HT=HT main effect, HA=HA main effect at $P<0.05$ by ANOVA.^a =different from WKY,^b =different from WKHT,^c =different from WKHA,^d =different from SHR at $P<0.05$ level by S-N-K.Underline indicates $P<0.10$ level by S-N-K to show trends in agreement with statistically significant results [14]. n=4 hearts in each group.

mRNA levels given in RQ values. Ct values for HPRT were subtracted from those of each listed gene to provide Ct, which was then normalized to value for WKY. Average fold expression of each gene relative to HPRT is given as Ct in parentheses.

Table 3

Gene (fold HPRT)	WKY	WKHT	WKHA	SHR	ANOVA
Cox3 (9.2)	1.00±0.03 ^{bcd}	1.65±0.04 ^a	1.33±0.11 ^a	1.46±0.03 ^a	HT, HT×HA
Dbh (<-15)	nd	nd	nd	nd	
Lox (-4.3)	1.00±0.03 ^{bc}	2.36±0.57 ^{ad}	2.50±0.54 ^{ad}	1.38±0.12 ^{bc}	HT×HA
Lox1l (-2.5)	1.00±0.05 ^{bc}	0.59±0.09 ^{acd}	1.30±0.03 ^{abd}	1.01±0.11 ^{bc}	HT, HA
Lox12 (-2.2)	1.00±0.01 ^{cd}	0.86±0.04 ^{cd}	1.87±0.30 ^{abd}	1.45±0.07 ^{abc}	HT, HA
Sod1 (2.7)	1.00±0.04 ^c	0.94±0.04 ^c	1.89±0.20 ^{abd}	1.12±0.03 ^c	HT, HA, HT×HA
Tyr (<-15)	nd	nd	nd	nd	
Mb (6.4)	1.00±0.01	1.05±0.07	1.02±0.08	0.93±0.11	
Fxyd1 (3.3)	1.00±0.01	1.12±0.01	1.18±0.16	0.97±0.17	
Maoa (0.1)	1.00±0.05 ^{bd}	1.78±0.40 ^{acd}	0.79±0.37 ^b	0.51±0.03 ^{ab}	HA, HT×HA
Maob (-4.3)	1.00±0.02 ^c	1.01±0.27 ^c	1.48±0.31 ^{abd}	1.16±0.04 ^c	HA

nd=effectively not detected due to Ct < -15 relative to HPRT. HT=HT main effect at $P<0.05$, HA=HA main effect at $P<0.05$, HT×HA=HT-HA interaction at $P<0.05$.

^a =different from WKY,

^b =different from WKHT,

^c =different from WKHA,

^d =different from SHR at $P<0.05$ level by S-N-K.

Underline indicates $P<0.10$ level to show trends in agreement with statistically significant results [14]. n=4 in each group.