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Deep transcriptomic profiling of Dahl salt-sensitive rat kidneys with mutant form of *Resp18*

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Abstract

Expression of Regulated endocrine specific protein 18 (**Resp18**) is localized in numerous tissues and cell types; however, its exact cellular function is unknown. We previously showed that targeted disruption of the *Resp18* locus in the Dahl SS (**SS**) rat (*Resp18*^{mutant}) results in higher blood pressure (**BP**), increased renal fibrosis, increased urinary protein excretion, and decreased mean survival time following a chronic (6 weeks) 2% high salt (**HS**) diet compared with the SS rat. Based on this prominent renal injury phenotype, we hypothesized that targeted disruption of *Resp18* in the SS rat promotes an early onset hypertensive-signaling event through altered signatures of the renal transcriptome in response to HS. To test this hypothesis, both SS and *Resp18*^{mutant} rats were exposed to a 7-day 2% HS diet and BP was recorded by radiotelemetry. After a 7-day exposure to the HS diet, systolic BP was significantly increased in the *Resp18*^{mutant} rat compared with the SS rat throughout the circadian cycle. Therefore, we sought to investigate the renal transcriptomic response to HS in the *Resp18*^{mutant} rat. Using RNA sequencing, *Resp18*^{mutant} rats showed a differential expression of 25 renal genes, including upregulation of *Ren.* Upregulation of renal *Ren* and other differentially expressed genes were confirmed via qRT-PCR. Moreover, circulating renin activity was significantly higher in the *Resp18*^{mutant} rat compared with the WT SS rat after 7 days on HS. Collectively, these observations demonstrate

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6.Conflict of Interest Statement

None

that disruption of the *Resp18* gene in the SS rat is associated with an altered renal transcriptomics signature as an early response to salt load.

Keywords

Resp18; Renin; RNA Seq; Dahl SS rat; Blood Pressure

1. Introduction

The interplay among genetic, behavior, and environmental factors plays a vital role in the development and progression of hypertension, also known as high blood pressure (**BP**). It is well established that upregulation of the renin-angiotensin-aldosterone system (**RAAS**) pathway increases BP and is a major contributor to water and electrolyte homeostasis [1]. However, advancements in technology provide us with new tools (gene-editing) to uncover additional genes associated with hypertension. The Dahl salt-sensitive (SS) rat is a well-established and clinically relevant experimental model for the study of salt-induced hypertension and renal failure [2]. Quantitative trait locus (**QTL**) mapping studies conducted in the SS rat have led to the identification of many genetic loci responsible for salt-induced hypertension and renal disease [3]. One such QTL study identified a genomic segment on rat chromosome 9 containing a potential candidate gene for BP and proteinuria, the Regulated Endocrine Specific Protein-18 (*Resp18*) [4]. Resp18 expression has been identified in numerous tissues and cell types; however, the precise function of this novel endocrine protein is currently unknown [5].

To validate *Resp18's* candidacy as causal of hypertension, $Resp18^{mutant}$ rats were generated by targeted disruption of the *Resp18* locus in the SS rat [2]. Our previous study showed that the $Resp18^{mutant}$ rat maintained on a high salt (**HS**) diet (2% NaCl) for 6 weeks exhibited an increase in BP, renal fibrosis, proteinuria and reduced mean survival time [2]. Furthermore, we also observed a significant increase in renal pro-fibrotic gene expression, including Collagen type 1 (***Col1a1***), Collagen type 3 (***Col3a1***), and transforming growth factor beta (***Tgf-β***) in the $Resp18^{mutant}$ rat [2]. From this study, we concluded that *Resp18* gene function is vital in BP regulation by modulating proper kidney homeostasis during salt loading.

Based on these studies we concluded that *Resp18* gene function is vital in BP regulation by modulating proper kidney homeostasis upon excess salt loading. Thus, the purpose of this study was to test the hypothesis that short-term exposure to a 2% HS diet (7-day) in the $Resp18^{mutant}$ rat, which exhibits a significant increase in BP in response to a chronic salt load, promotes an early onset hypertensive-signaling event through altered signatures of the renal transcriptome in response to HS.

2. Materials and Methods

2.1. Rats

Male Dahl salt-sensitive/Mcw (SS) and $Resp18^{mutant}$ rats were concomitantly bred and raised as separate colonies fed a low salt diet (0.3% NaCl; Harlan Teklad diet 7034) until 6 weeks of age [2]. After 6 weeks of age, the diet was switched to HS (2% NaCl;

Harlan Teklad diet 94217) for 7 days. After 7 days of HS, the kidneys were collected and immediately frozen in liquid nitrogen. All rats were kept on a 12:12-h light-dark cycle in a climate-controlled room. Rat chow and water were provided ad libitum. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Toledo, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and followed ARRIVE guidelines.

2.2. Measurement of blood pressure using radiotelemetry

At five weeks of age, SS and *Resp18*^{mutant} rats were anesthetized using isoflurane and surgically implanted with radiotelemetry transmitters, as previously described [2]. The rats were individually housed after the surgery and allowed to recover for 3 days. Using DSI software and equipment (https://www.datasci.com/), BP data were collected every 5 min, starting a day prior to HS until end of study (after 7 days HS) and analyzed using Dataquest A.R.T 4.2 software.

2.3 RNA Sequencing

See in Supplemental Materials and methods

2.4 Validation of analysis of RNA- Seq data by qRT-PCR

See in supplemental Materials and methods

2.5 Measurement of serum Renin activity

Renin activity was measured in serum obtained from SS and *Resp18*^{mutant} rats to determine renin activity prior to and after exposure to a 7-day HS diet. Renin activity was measured using a renin assay kit (MAK157-1 KT -Sigma Aldrich, USA), as per manufacturer's instructions.

2.6 Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed by Student's t-test or two-way ANOVA (Sidak test), as appropriate, with P value of <0.05 was used as a threshold for statistical significance. Figures were generated with GraphPad Prism software (version 7; GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. *Resp18*^{mutant} rats maintain a significant increase in systolic blood pressure in response to a 7-day HS diet compared with SS counterparts

In the current study, SBP was significantly increased at 7 weeks of age in *Resp18*^{mutant} rats on a 0.3% salt (**LS**) diet, and in *Resp18*^{mutant} rats exposed to 7 days of a HS diet when compared with SS rats (Fig 1A), regardless of light or dark phase of the circadian cycle. DBP was increased in *Resp18*^{mutant} rats on LS but not in response to the HS diet (Fig. 1B). The mean arterial pressure (MAP) in the *Resp18*^{mutant} was not increased by HS diet, which was the case in SS rats, but the MAP remained significantly higher in the *Resp18*^{mutant} than SS rats (Fig 1C).

3.2. Transcriptome analysis of kidneys from the *Resp18*^{mutant} rat

As shown on the heat map visualizing results from our RNA-Seq analysis, the renal transcriptome response to a 7-day HS diet was significantly different upon comparison of the *Resp18*^{mutant} rat to the SS rat (Fig 2A and Supplementary Table 2). Using a volcano plot to infer the overall distribution of differentially expressed renal genes, 25 genes were differentially expressed between the *Resp18*^{mutant} and SS rats with the threshold set at a p-value <0.05 and with all biological variation removed by DESeq (Fig 2B). Out of the 25 genes, expressions of 12 genes were upregulated and 13 genes were downregulated (Fig 2B). Venn diagram analysis of the RNA-Seq data showed 370 uniquely expressed genes in kidneys from *Resp18*^{mutant} rat and 294 uniquely expressed genes in kidneys from the SS rat (Fig 2C).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to understand the biological relevance of the DEG-enriched pathways. The eight pathways significantly upregulated in the *Resp18*^{mutant} rat were associated with transcriptional mis-regulation in cancer, *Staphylococcus aureus* infection, renin secretion, renin-angiotensin system, p53 signaling, endocrine, and other factor-regulated calcium reabsorbing, cell adhesion molecule (**CAMs**), and ABC transporters pathways (Fig 2D). KEGG pathway analysis also revealed three pathways correlated with significantly downregulated genes that included RNA transport, glutamatergic synapse, and FoxO signaling pathway (Fig 2D).

To validate our RNA-Seq results, qRT-PCR was used to analyze a subset of genes that demonstrated a significant differential expression. Through qRT-PCR analysis, we validated the differential expressions of numerous up-regulated and down-regulated genes in the HS-exposed *Resp18*^{mutant} rat (Fig 3A&B). We also found eight novel transcripts that were differentially expressed between *Resp18*^{mutant} and SS rats.

3.3. Circulating renin activity is increased in the *Resp18*^{mutant} rat in response to 7-day salt load

The renin-angiotensin-aldosterone system (RAAS) plays a pivotal role in regulating water and electrolyte balance and BP [6]. RNA-Seq analysis with validation by qRT-PCR demonstrated that renal renin gene expression was significantly upregulated in the *Resp18*^{mutant} rat compared with the SS rat. Circulating renin activity before HS diet was not different between *Resp18*^{mutant} and SS rat (Fig 4A). However, after 7days on HS, circulating renin activity was significantly increased in *Resp18*^{mutant} compared with SS rat (Fig 4B).

4. Discussion

Based on our previous findings, which suggest that the *Resp18* gene plays an essential role in decelerating the progression of salt-induced hypertension and renal injury, [2] this study tested the hypothesis that 7 days of HS starting at 6 weeks of age promotes early transcriptomic changes in the kidney of the *Resp18*^{mutant} rat. RNA-Seq revealed 25 renal DEG's between *Resp18*^{mutant} and SS rats. In total, 13 genes were downregulated, and 12 genes were upregulated and were confirmed through qRT-PCR analysis. Among these DEGs, six are closely associated with BP and included *Homer1* [7], *Selplg* [8], *Abca8a* [9],

Dpep1 [10], *Calb1* [11], and *Ren* [12]. Yet, for some, the relevance for altered expression confirmed by qRT-PCR was not immediately clear.

Homer is upregulated in coronary artery disease and plays a role in the regulation of G protein-coupled receptors [13]. Yet, intra-renal *Homer1* gene expression was downregulated in the *Resp18*^{mutant} rat after a 7-day exposure to HS. Selectin P Ligand (*Selplg*) gene expression was upregulated in the kidney of the *Resp18*^{mutant} rat, in response to HS. Decreased Selplg binding capacity, can lead to fewer leukocyte—endothelium and leukocyte—platelet complexes and potentially reduce the risk of stroke [8]. Single nucleotide polymorphisms (SNPs) within the *ABCC8* gene, a member of the superfamily of ATP-binding cassette (ABC) transporters, were found to be associated with pulmonary arterial hypertension [14]. *Dpep1,* another differentially expressed gene encodes for a membrane-bound enzyme in the kidney, which is involved in the hydrolysis of dipeptides, such as glutathione and other similar proteins [15]. Furthermore, Dpep1 regulates leukotriene activity by catalyzing the conversion of leukotriene D4 to leukotriene E4 [16]. Leukotrienes are pro-inflammatory mediators and are also potential contributors to hypertension [17]. Calb1 plays a role in Ca^{2+} transport and anti-calcification process. In the kidney, Calb1 regulates renal tubular Ca^{2+} reabsorption [18], along with other partners such as Trpv5, Trpv6, Pmca1b calcium pump, and Ncx1 exchanger [19]. Interestingly, in our studies we found that Trpv5 was upregulated in *Resp18*^{mutant} rat kidneys. A SNP identified in the TRPV5 gene is closely associated with stone multiplicity in calcium nephrolithiasis patients [20]. Another differentially expressed gene, such as Adamts like 2, a secreted glycoprotein binds to the cell surface and extracellular matrix, also interacts with latent transforming growth factor beta binding protein 1 [21]. Furthermore, through substitution mapping studies, genetic loci, including genes such as *Pfdn4* [22], *Cpne7* [23], *Eif4a2* [24], and *Unc79* [25], are significantly associated with BP, stress-related neuroendocrine reactivity, and urinary albumin excretion quantitative trait locus.

In our study, we chose to focus on the role of renin, well known for its role in BP regulation and renal function in the SS rat, a low renin model of hypertension. KEGG pathway analysis revealed upregulation of the intra-renal *Ren* gene expression in the *Resp18*^{mutant} rat versus the SS rat on HS diet. Exposure to a HS diet suppresses serum renin activity and circulating angiotensinogen in the SS rat, suggesting that the SS rat is a model of low renin hypertension [26]. Yet, Kobori et al., reported that intrarenal angiotensinogen is increased in the SS rat on HS, indicative of tissue-specific inappropriate activation of the intrarenal RAAS [26]. In our study, circulating renin activity prior to HS in the *Resp18*^{mutant} rat was comparable to the SS rat. However, following a 7-day exposure to HS diet circulating renin activity was significantly elevated in the *Resp18*^{mutant} rat, an increase that also correlated with a significant increase in SBP and intra-renal *Ren* gene expression compared with the SS rat. Whether intra-renal *Ren* gene expression was elevated prior to HS is not yet known. An increase in *Ren* gene expression and circulating levels of renin are known to promote hypertension and other cardiovascular and renal complications [27]. Thus, the factors that contribute to increased BP in the *Resp18*^{mutant} rat on LS remain unknown although up-regulation of intra-renal *Ren* is not yet ruled out, as a contributory factor. Collectively, these data suggest a differential response of intra-renal *Ren* gene expression in the *Resp18*^{mutant} rat compared with the SS rat on HS diet. Whether upregulation of

other components of the classical intrarenal RAAS in the *Resp18*^{mutant} rat correlates with increased circulating and intra-renal renin is not yet known. However, these findings suggest that exposure to a HS diet promotes an early transcriptomic response that favors the rise in BP and altered gene expression of known factors, such as renin and unknown players (eight novel transcripts). The effect of systemic activation of the RAAS on secondary immune activation and inflammation in the *Resp18*^{mutant} rat is also unclear and remains a focus of ongoing studies.

Little is known regarding the cellular function of *Resp18*; however, using a bioinformatics approach, it was discovered that RESP18 shares sequence homology with the luminal region of IA-2, a DCV (dense core vesicle) transmembrane protein involved in insulin secretion [28]. Furthermore, RESP18 expression is found in the lumen of DCVs [29], collectively suggesting that *Resp18* may play a role in hormone or peptide secretion. Based on findings from our study, *Resp18* may play a role in the control of renin secretion and the regulation of BP in response to a HS diet in the *Resp18*^{mutant} *rat.* To date, there is only one FDA-approved direct renin inhibitor (aliskiren) on the market for the treatment of hypertension [30]. Additional studies will be required to validate the relationship between Resp18 and renin synthesis and secretion, and activation of the RAAS and its downstream components in the onset and progression of increased BP and hypertension.

The SS rat is a well-established experimental model utilized for study of salt-sensitive hypertension. Historically considered a model of low renin, the SS rat is implicated to mimic the etiology of hypertension in individuals non-responsive to ACE inhibition, most likely due to suppression of the RAAS [26]. In our study, circulating renin activity was elevated in response to HS in the *Resp18*^{mutant} rat, compared with the SS rat, suggesting that mutation of *Resp18* alters the ability of salt to suppress the systemic RAAS. Whether intra-renal *Ren* gene expression is elevated in the *Resp18*^{mutant} rat compared with SS rat, prior to HS is not yet known. Yet, the sustained increase in BP regardless of salt intake implicates a critical role for Resp18 in the long-term control of BP. Moreover, our findings suggest that increased BP in the *Resp18*^{mutant} rat that persists in response to salt load may involve “global” in addition to tissue-specific actions of the RAAS. Thus, insight using this novel model of cardiorenal risk may lead to the identification of yet unexplored therapeutics for the treatment of high BP that occurs even in the absence of salt load.

In summary, targeted disruption of the *Resp18* locus in SS rats leads to a hypertensive phenotype even after short-term exposure (7-day) to a HS diet. RNA-Seq analysis, confirmed by qRT-PCR, demonstrated up-regulation of renal *Ren* gene expression. However, unlike the Dahl SS rat, our study showed a significant increase in circulating renin activity in the *Resp18*^{mutant} rat. *Resp18* shares sequence homology with IA-2 a, DCV involved in the secretion of hormones and neurotransmitters. Based on our current study, we speculate that *Resp18* gene plays a pivotal role in systemic and intra-renal renin synthesis and/or secretion, and thus, regulation of BP upon exposure to a HS diet. One limitation of our study involved the study of male but not female *Resp18*^{mutant} rats. It is well established that sex alters the phenotypic response to HS in the SS rat. Whether sex alters BP in response to LS or HS in the female *Resp18*^{mutant} rat is not yet known. Future studies will determine the phenotypic response in the female *Resp18*^{mutant}. Moreover, aging may be another variable that also

influences the progression and severity of cardiorenal risk that occurs even under conditions of reduced salt intake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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7. References

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Highlights

- The study highlights the physiological role of Resp18, a novel player in salt-induced hypertension.
- The study emphasizes the early transcriptomic signature of genes involved in HS-induced hypertension in Resp18 mutant rat kidneys.
- This study also shows that targeted disruption of the Resp18 locus in Dahl SS rat (*Resp18mutant*) up-regulates RAAS.
- Our study highlights the use of this novel model cardiorenal risk may lead to the identification of yet unexplored therapeutic targets for the treatment of high BP that occurs even in the absence of salt load.

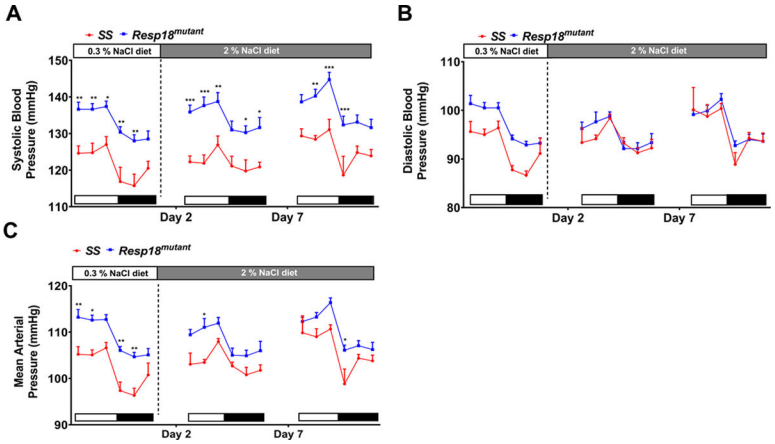

Figure 1. *Resp18*^{mutant} rats maintain elevated blood pressure compared with wild-type SS rats after exposure to 2% high salt (HS) diet.

Systolic blood pressure (A), diastolic blood pressure (B), and mean arterial pressure (MAP) (C) were measured using radiotelemetry in SS and *Resp18*^{mutant} rats (n = 8/group). White bars represent light cycle and black bars represent dark cycle. Data presented are the 4 h average of recordings obtained every 5 min continuously for 24 h. Data represent mean±SEM. Two-way ANOVA with Holm-Šídák was used for comparison. *p<0.05, **p<0.01 ***p<0.001.

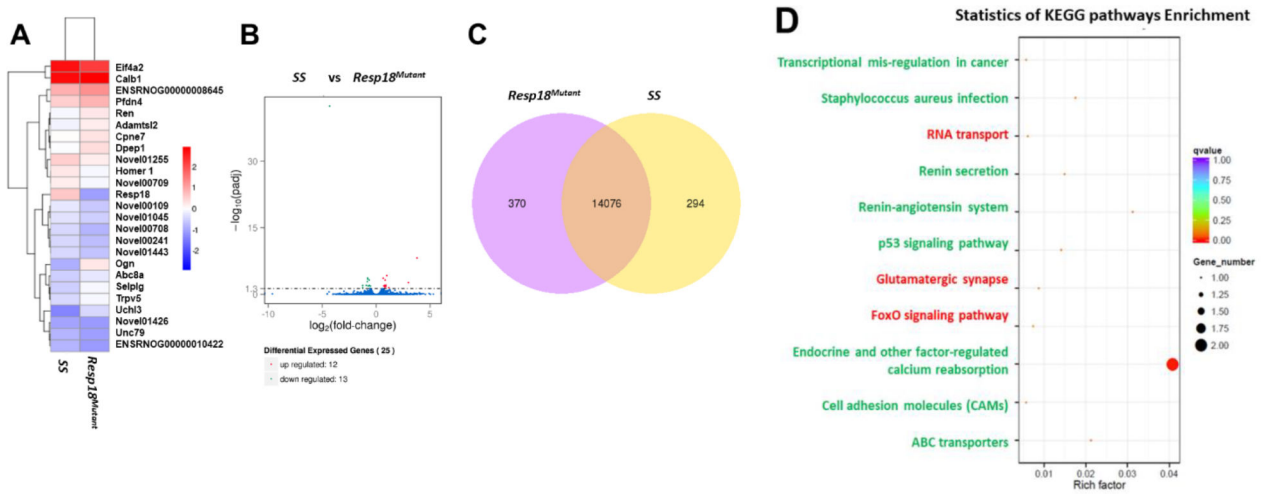

Figure 2. Transcriptome response to a 7-day exposure to a 2% high salt (HS) diet is altered in the *Resp18*^{mutant} rat kidney, relative to wild-type SS rat kidney:

(A) Heat map results of a FPKM cluster analysis, using the $\log_{10}$ (FPKM+1) value. Red denotes renal genes with high expression levels; blue denotes renal genes with low expression levels in the *Resp18*^{mutant}. (B) Volcano plots x-axis shows the fold-change in gene expression between samples (-$\log_{10}$(padj) and the y-axis shows the statistical significance of the difference ($\log_2$ (fold-change). Significant up- and down-regulated genes are highlighted in red and green, respectively. Genes which are not differentially expressed are in blue. (C) Venn diagram analysis with yellow representing uniquely expressed renal genes in the SS rat and purple representing uniquely expressed renal genes in the *Resp18*^{mutant} rat (D) KEGG analysis of differential gene expression in kidneys. Green text represents pathways that correlate with upregulated genes; red text represents pathways that correlate with downregulated genes. Dot size represents the number of different genes and color represents the q-value.

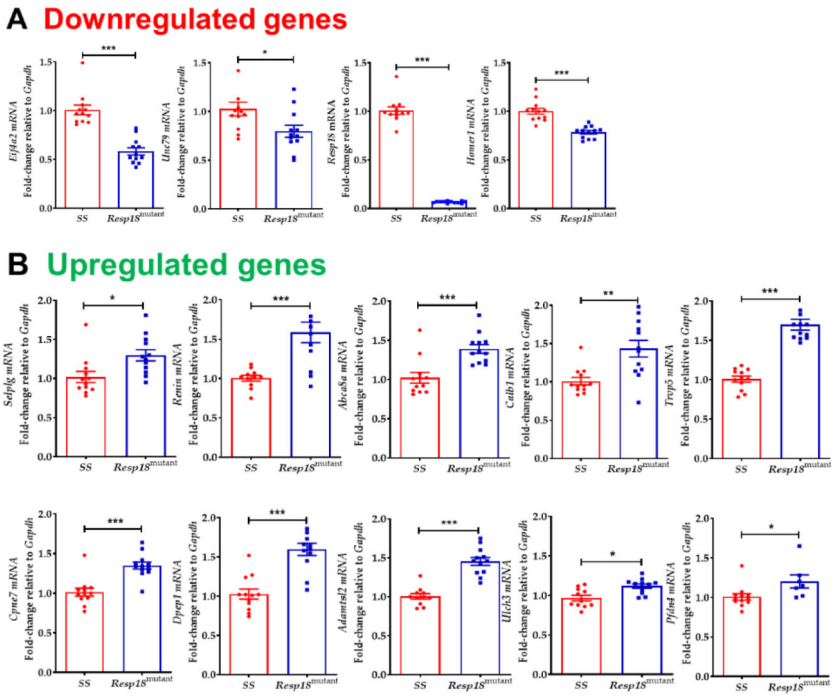

Figure 3. qRT-PCR validation of RNA seq:

(A) downregulated or (B) upregulated in *Resp18*^{mutant} rats compared with the SS rats (n=4/grp in duplicates). Data represent mean±SEM. Statistical significance was calculated by unpaired two-tailed t-test *p<0.05, **p<0.01 ***p<0.001.

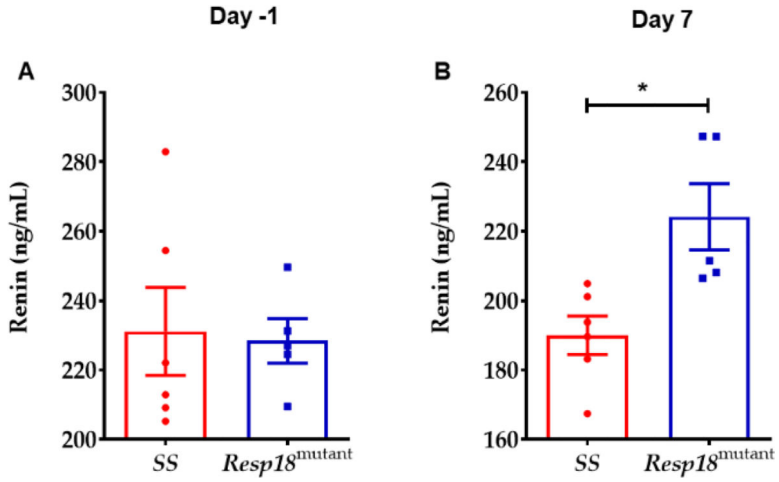

Figure 4: Renin levels in the *Resp18*^{mutant} rat after a 7-day exposure to a 2% high salt (HS) diet: (A) Serum samples collected from *Resp18*^{mutant} and SS rats prior to exposure to the HS (n=5–6/grp) and after 7 days on HS (n=5–6/grp). Data represent mean±SEM. Statistical significance was calculated unpaired two-tailed t-test **p<0.05.