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Corresponding Author: Dr. You-Lin Tain, M.D., Ph.D.

Corresponding Author's Institution: Kaohsiung Chang Gung Memorial Hospital

First Author: You-Lin Tain, M.D., Ph.D.

Order of Authors: You-Lin Tain, M.D., Ph.D.; chien-Ning Hsu, PhD; Ching-Yuang Lin, MD, PhD; Li-Tung Huang, MD; Ying-Tung Lau, PhD

Abstract: Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, decreases NO synthesis. Plasma ADMA concentrations increase markedly in hypertension. We tested whether the development of hypertension and the increases in ADMA in spontaneously hypertensive rats (SHR) are prevented by aliskiren, a renin inhibitor. Male SHRs and normotensive Wistar Kyoto (WKY) control rats, aged 4 weeks (pre-hypertensive stage), were assigned to 4 groups: untreated SHRs and WKY rats, and SHRs that received oral aliskiren 10 and 30 mg/kg/day for 6 weeks. All rats were sacrificed at age 10 weeks. Blood pressure decreased at age 6, 8, and 10 weeks in SHRs that received high-dose aliskiren. Aliskiren mitigated the increases in plasma ADMA in SHRs. Renal ADMA levels were lower in SHRs that received high-dose aliskiren versus SHRs. SHRs experienced decreased plasma and kidney L-Arg-to-ADMA ratios versus control rats, which were reverted by 30 mg/kg aliskiren. Renal cortical neuronal NOS-α and -β levels increased in SHRs fed with high-dose aliskiren. Early aliskiren treatment mitigates increases in ADMA, restores L-Arg-to-ADMA ratios, enhances neuronal NOS-α, prevents decreased nNOS-β levels in the kidney—which might restore NO bioavailability and contribute to the decrease of blood pressure in young SHRs. Our findings suggest that aliskiren is a therapeutic agent for prehypertension that regulates the ADMA/NO pathway.

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Aliskiren prevents hypertension and reduces asymmetric dimethylarginine in young spontaneously hypertensive rats

You-Lin Tain[a*], Chien-Ning Hsu[b], Ching-Yuang Lin[c], Li-Tung Huang[a], and Ying-Tung Lau[d]

[a]Departments of Pediatrics and [b]Pharmacy, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan

[c]College of Medicine, China Medical University, Clinical Immunological Center, and Division of Pediatric Nephrology, China Medical University Hospital, Taichung, Taiwan

[d]Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, and Chang Gung Institute of Technology, Taoyuan, Taiwan

Corresponding author: You-Lin Tain, MD, PhD

Department of Pediatrics, Chang Gung Memorial Hospital-Kaohsiung Medical Center

123 Dabi Road, Niausung, Kaohsiung 833, Taiwan

E-mail address: tainyl@hotmail.com

Tel: +886-975056995, Fax: +886-7-7338009

Abstract

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, decreases NO synthesis. Plasma ADMA concentrations increase markedly in hypertension. We tested whether the development of hypertension and the increases in ADMA in spontaneously hypertensive rats (SHR) are prevented by aliskiren, a renin inhibitor. Male SHRs and normotensive Wistar Kyoto (WKY) control rats, aged 4 weeks (pre-hypertensive stage), were assigned to 4 groups: untreated SHRs and WKY rats, and SHRs that received oral aliskiren 10 and 30 mg/kg/day for 6 weeks. All rats were sacrificed at age 10 weeks. Blood pressure decreased at age 6, 8, and 10 weeks in SHRs that received high-dose aliskiren. Aliskiren mitigated the increases in plasma ADMA in SHRs. Renal ADMA levels were lower in SHRs that received high-dose aliskiren versus SHRs. SHRs experienced decreased plasma and kidney L-Arg-to-ADMA ratios versus control rats, which were reverted by 30 mg/kg aliskiren. Renal cortical neuronal NOS-α and -β levels increased in SHRs fed with high-dose aliskiren. Early aliskiren treatment mitigates increases in ADMA, restores L-Arg-to-ADMA ratios, enhances neuronal NOS-α, prevents decreased nNOS-β levels in the kidney—which might restore NO bioavailability and contribute to the decrease of blood pressure in young SHRs. Our findings suggest that aliskiren is a therapeutic agent for prehypertension that regulates the ADMA/NO pathway.

Key Words: asymmetric dimethylarginine, nitric oxide, hypertension

1 Introduction

The prevalence of prehypertension and hypertension in children and adolescents is increasing (McCrindle, 2010). Children with prehypertension are at increased risk of developing hypertension and end-organ damage in adulthood. It is unclear whether a particular antihypertensive medication is beneficial for certain high-risk children (e.g., those with chronic kidney disease) to prevent the development of hypertension and end-organ damage (Schunkert, 2006).

The renin-angiotensin-aldosterone system (RAAS) regulates blood pressure. Currently, ACE inhibitors and angiotensin AT_1 receptor blockers constitute the basis of antihypertensive therapy (Paulis and Unger, 2010). Treatment of young spontaneously hypertensive rats (SHRs) with ACE inhibitors prevents full-blown hypertension and cardiovascular hypertrophy (Harrap et al., 1990). Similarly, ACE inhibitors and angiotensin AT_1 receptor blockers have prevented the transition from prehypertension to hypertension in clinical trials (Julis et al., 2006; Luders et al., 2008).

Because the RAAS cascade begins with the renin and because aliskiren, a renin inhibitor, is similar to other antihypertensive agents for blood pressure reduction (Paulis and Unger, 2010; Sanoski, 2009), we aimed to determine the effects of aliskiren in prehypertension by using the young SHR model.

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), reduces NO production, thereby causing endothelial dysfunction, vasoconstriction, and blood pressure elevation (Boger et al., 2009). Plasma concentrations of ADMA increase markedly in patients with hypertension and end-organ damage and in high-risk subgroups with prehypertension (Wilcox, 2005). Such increases are reduced by ACE inhibitors and angiotensin AT_1 receptor blockers (Beltowski and Kedra, 2006), but the underlying mechanisms of this phenomenon remain unclear. Aliskiren dose-dependently decreases blood pressure in SHRs (Wood et al., 2005), but whether this effect is due to its lowering ADMA has not been examined.

We recently observed that melatonin concurrently prevents increases in ADMA and hypertension in young SHRs (Tain et al., 2010a). In addition, we noted that reactive oxygen species raise ADMA levels by inhibiting dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that metabolizes ADMA (Tain et al., 2010b). Since aliskiren can reduce the production of reactive oxygen species (Sanoski, 2009), it might cause less inhibition on DDAH to increase ADMA metabolism. The resulting reduction in ADMA might lead to greater NO production and reduce hypertension.

To determine whether aliskiren prevents the increases in ADMA and protects

against the development of hypertension, we treated young SHRs with aliskiren, using 2 dosages that have been used in adult SHRs to reduce blood pressure. In addition, because blood pressure can be regulated by neuronal NOS (nNOS) and renin in the macula densa and because aliskiren enhances endothelial NOS (eNOS) expression to restore NO (Imanishi et al., 2008), we also evaluated whether aliskiren preserves renal cortical nNOS and eNOS expression.

2 Materials and Methods

2.1 Animals and pharmacological treatment

This study was approved and performed per the Guidelines for Animal Experiments of Chang Gung Memorial Hospital and Chang Gung University. The treatment of animals conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Three-week-old male SHRs and normotensive Wistar Kyoto (WKY) control rats were obtained (BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) and given free access to tap water and standard rat chow.

Three groups of 4-week-old male SHRs (N = 8 per group) were sacrificed after a 6-week treatment: Group 1, untreated SHRs; Group 2 (SHR+A10), SHRs that received oral aliskiren 10 mg/kg/day via gastric gavage (Novartis Pharmaceutical, New York, NY, USA); Group 3 (SHR+A30), SHRs that received oral aliskiren 30 mg/kg/day; and a control group of 4-week-old male WKY rats (N = 8).

Blood pressure was measured in conscious rats by an indirect tail-cuff method (BP-2000; Visitech Systems Inc., Apex, NC, USA) after the animals were systematically trained at age 4, 6, 8, and 10 weeks as previously described (Tain et al., 2010a). All rats were sacrificed at age 10 weeks. Heparinized blood samples were collected, and kidneys and hearts were harvested. Plasma NOx (NO_2^- + NO_3^-) level

was measured by the Griess reaction as previously described (Tain et al., 2007).

2.2 Detection of L-arginine and ADMA by HPLC

Plasma and tissue L-arginine and ADMA levels were measured by HPLC (HP series 1100; Agilent Technologies Inc., Santa Clara, CA, USA) with the OPA-3MPA derivatization reagent, as described (Tain et al., 2010a). The standards contained L-arginine and ADMA at 1–100 μM and 0.5–5 μM, respectively. The recovery rate was 90% to 105%. The tissue concentration was adjusted for protein concentration and expressed as μM/mg protein.

2.3 Western blot

Western blot analysis was performed as described (Tain et al., 2007; Tain et al., 2010a). We used the following antibodies: for protein arginine methyltransferase (PRMT)-1, rabbit anti-human PRMT-1 (1:200; Millipore, Billerica, MA, USA); for DDAH, goat anti-rat DDAH-1 (1:500, overnight incubation; Santa Cruz, Santa Cruz, CA, USA) and goat anti-rat DDAH-2 (1:100, overnight incubation; Santa Cruz); for nNOS-α, mouse monoclonal antibody (Santa Cruz); for nNOS-β, a rabbit polyclonal antibody (Affinity BioReagents, Golden, CO, USA); for eNOS, a mouse monoclonal antibody (1:250, 1-hour incubation; Transduction Laboratories). The bands of interest were visualized using ECL reagents (PerkinElmer, Waltham, MA, USA) and quantified by densitometry (Quantity One Analysis software; Bio-Rad), calculated as

the integrated optical density (IOD) minus the background value. The IOD was adjusted for Ponceau red staining (PonS) to correct for variations in total protein loading; protein abundance was expressed as IOD/PonS.

2.4 DDAH activity

DDAH activity was measured by colorimetric assay, which determines the rate of citrulline production, as optimized by us recently (Tain and Baylis, 2007). Kidney cortex was homogenized in sodium phosphate buffer. Tissue homogenates were preincubated with urease for 15 min, and 100 μl (2 mg) of homogenate was incubated with 1 mM ADMA for 45 min at 37°C. After deproteinization, the supernatant was incubated with color mixture at 60ºC for 110 min. Each sample was analyzed with a paired blank (that lacked ADMA) to prevent the effects of citrulline interference. The absorbance was measured by spectrophotometry at 466 nm. DDAH activity was expressed as micromolars of citrulline generated per gram of protein per minute at 37ºC.

2.5 Statistics

Results were expressed as mean ± S.E.M. Biochemical parameters were analyzed by 1-way ANOVA with post hoc LSD test. All analyses were performed using SPSS. $P < 0.05$ was considered statistically significant.

3 Results

The body weight decreased in the SHR groups that were treated with aliskiren (WKY: 270.6 ± 3.0g; SHR: 271.5 ± 3.5g; SHR+A10: 242.3 ± 4.1g; SHR+A30: 243.7 ± 3.0g, $P < 0.05$). The heart weight-to-body weight ratio did not differ between the 4 groups, but the kidney weight-to-body weight ratio in SHRs (0.5 ± 0.02) increased compared with WKY rats (0.45 ± 0.01, $P < 0.05$), which was prevented by both doses of aliskiren (SHR+A10: 0.43 ± 0.01; SHR+A30: 0.42 ± 0.01, both $P < 0.05$).

Baseline mean arterial pressure was similar in the 4 groups, and mean arterial pressure increased in untreated SHRs over 6 weeks compared with WKY (Fig. 1). There was a greater reduction in mean arterial pressure in the SHR+A30 group versus the SHR+A10 group at age 6, 8, and 10 weeks. As shown in Fig. 1, mean arterial pressure decreased progressively in the SHR+A30 group but not in the SHR+A10 group, suggesting that aliskiren reduces blood pressure dose dependently.

Plasma NOx levels were comparable in WKY rats (28 ± 2.9 μmol) and SHRs (30.4 ± 2.3 μmol). Plasma NOx levels increased significantly in the SHR+A30 group (41.7 ± 3.3 μmol) but not in the SHR+A10 group (29 ± 2.1 μmol).

As shown in Table 1, aliskiren significantly limited the increases in plasma ADMA in SHRs. L-arginine level in the kidney did not differ between the 4 groups. Renal ADMA levels were lower in the SHR+A30 group than in SHRs and SHR+A10

animals. Because ADMA and L-arginine compete for NOS, we analyzed the L-arginine-to-ADMA ratio to determine NO bioavailability. SHRs had decreased L-arginine-to-ADMA ratios in the plasma and kidney compared with WKYs, which were prevented by 30 mg/kg aliskiren.

Next, the levels of PRMT-1 (ADMA-synthesizing enzymes) in the kidney did not differ between the 4 groups (Fig. 2B). Similar to our previous findings (Tain et al., 2010a), the expression of DDAH-1, an ADMA-metabolizing enzyme, did not differ between the 4 groups (Fig. 2C). However, aliskiren significantly upregulated DDAH-2 in SHR kidneys (Fig. 2D). In addition, renal DDAH activity decreased in SHRs, which was prevented by high-dose aliskiren (Fig. 2E).

eNOS levels in the kidney were similar in the 4 groups (Fig. 3B). Renal cortical nNOS-α levels were higher in the SHR+A30 group than in the other groups (Fig. 3C). nNOS-β levels in the kidney declined in SHRs compared with WKY rats, which was prevented by 30 mg/kg aliskiren (Fig. 3D).

4 Discussion

This study demonstrates that the human renin inhibitor aliskiren prevents the development of hypertension in young SHRs. Based on our findings, aliskiren reduces ADMA levels, restores L-arginine-to-ADMA ratios, enhances renal cortical nNOS-α and recovers decreased nNOS-β levels. These evidences proposed a link between renin inhibition, augmentation of NO bioavailability, and reduction of blood pressure. Although some studies demonstrated that ACE inhibitors and angiotensin AT_1 receptor blockers might induce NO bioavailability (Luft and Weinberger, 2008), few studies have examined whether renin inhibitors can regulate the NO pathway. In addition to blockage of the active site of renin and the subsequent decline in plasma renin activity and angiotensin II (Luft and Weinberger, 2008), we demonstrated for the first time the antihypertensive effects of aliskiren are, at least in part, by regulating ADMA/NO pathway.

Although renin is species-specific with regard to its substrate and although aliskiren favors human renin over non-human renins (Luft and Weinberger, 2008), blood pressure decreased markedly with both aliskiren doses in the last 4 weeks of treatment. High-dose aliskiren (30 mg/kg/day) significantly reduced mean arterial pressure over time. Our data are consistent with a previous study that reported that aliskiren dose-dependently lowers blood pressure in adult SHRs with established

hypertension (Wood et al., 2005). In addition, both aliskiren doses ameliorated renal hypertrophy. Consistent with growing evidence that blockade of the RAAS prevents hypertensive end-organ damage (Duprez, 2006), our data suggest that early treatment with aliskiren affords renoprotection.

Our previous report indicated that increases in ADMA levels in the plasma and kidneys precede hypertension in SHR. Lowering ADMA levels to shift the NO/ reactive oxygen species balance toward increased NO bioavailability during the prehypertensive stage can prevent the development of hypertension in young SHRs (Tain et al., 2010a). Consistent with our previous findings, we observed an approximately 50% increase in plasma ADMA concentration and a roughly 20% increase in the renal ADMA levels in 10-week-old SHRs compared with age-matched WKY rats. Notably, in addition to its significant blood pressure-lowering effects, 30 mg/kg/day aliskiren reduced the plasma ADMA increases by approximately 20% after 6 weeks of treatment and lowered renal ADMA concentrations in SHRs to levels that were lower than in WKY rats.

ADMA is being targeted therapeutically to prevent cardiovascular morbidity and mortality (Beltowski and Kedra, 2006); however, specific ADMA-lowering agents do not exist. Treatment with ACE inhibitors and angiotensin AT_1 receptor blockers has been reported to lower plasma ADMA levels by 10% to 24% (Beltowski and Kedra,

2006). Our study has demonstrated that renin inhibitors have ADMA-lowering effects and similar to other RAAS-blocking agents. In addition, we observed that high-dose aliskiren reduces ADMA levels not only in plasma but also in the kidneys. Because ADMA induces glomerular and vascular fibrosis (Mihout et al., 2010), we must determine whether the reductions in tissue ADMA concentration that are caused by aliskiren are beneficial in protecting against end-organ damage.

We recently observed that the accumulation of ADMA in the kidneys is due primarily to impaired ADMA catabolism, secondary to decreased DDAH expression and activity (Tain et al., 2010a). In this study, we found high-dose aliskiren increased DDAH-2 levels, slowing the accumulation of ADMA in SHR kidneys. In addition, aliskiren prevented the decline in renal DDAH activity in SHRs. Furthermore, we observed that high-dose aliskiren partially prevented decreases in the L-arginine-to-ADMA ratio in plasma and markedly increased the L-arginine-to-ADMA ratio in kidney. Because improvements in L-arginine-to-ADMA ratio by angiotensin AT_1 receptor blockers prevent hypertension and kidney damage in fawn-hooded hypertensive rats (Chen et al., 2010), our findings suggest that the protective effects of aliskiren on hypertension and kidneys are due in part to restore L-arginine-to-ADMA ratios.

Like melatonin (Tain et al., 2010a), aliskiren decreases blood pressure and

ADMA concurrently. However, compared with melatonin, its ADMA-lowering effect is inferior and its blood pressure-lowering effect is superior. Apart from aliskiren's protective effects on ADMA and L-arginine-to-ADMA ratio, we observed that aliskiren enhances renal cortical nNOS, which might improve NO bioavailability and explain this discrepancy.

In the kidney cortex, nNOS abounds in the macula densa; it mediates renin synthesis, dilates afferent arterioles, and reduces sodium reabsorption (Tojo et al., 2006). Thus, renal nNOS/NO defects result in the constriction of afferent arterioles and impaired pressure natriuresis, leading to the development of hypertension and kidney damage. Welch et al. noted the dissociation between enhanced nNOS expression and defective NO function in the kidney cortex in SHRs (Welch et al., 2000); this discrepancy might be attributed to our findings—that increases in ADMA inhibit nNOS activity.

In addition, we recently identified 2 isoforms of nNOS (splice variants) in normal rat kidney (Smith et al., 2009). SHRs had slightly decreased renal cortical nNOS-α levels and significant lower nNOS-β levels, which were reverted by aliskiren. Because nNOS expression correlates with afferent arteriolar diameter (Tojo et al., 2006), aliskiren might shift the renal constrictor-dilator balance, which has been disturbed by activation of the RAAS in the SHR, toward relaxation. Regarding the

protein levels of eNOS, nNOS-β, and DDAH-2 in SHRs and WKY rats, there are not identical on this study and our previous observations (Tain et al., 2010a). These discrepancies may be related to differences in the rat age and low sample size to reach a sufficient statistical power.

5 Conclusions

In conclusion, early aliskiren treatment decreases ADMA, increases L-arginine-to-ADMA ratio, increases nNOS-α, restores decreased nNOS-β levels in the kidney, which might enhance NO bioavailability and contribute to prevent the development of hypertension in young SHRs. Although aliskiren ameliorates established hypertension, our data suggest that treatment with aliskiren in the prehypertensive stage prevents the development of hypertension later in life by regulating ADMA/NO pathway.

Acknowledgements

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Figure Legends

Fig. 1. Effects of aliskiren on mean arterial pressure in WKY rats and SHRs. *P < 0.05 vs. WKY; #P < 0.05 vs. SHR; §P < 0.05 SHR+aliskiren 10 mg vs. SHR+aliskiren 30 mg.

Fig. 2. Representative western blots of (A) PRMT-1 (~42 kDa), DDAH-1 (~34 kDa), and DDAH-2 (~30 kDa) in WKY rats and SHRs at age 10 weeks. Relative abundance of renal cortical (B) PRMT-1, (C) DDAH-1, and (D) DDAH-2 was quantified. N = 8/group; A10 = SHRs treated with aliskiren 10 mg/kg/day; A30 = SHRs treated with aliskiren 30 mg/kg/day. (E) Effect of aliskiren on renal DDAH activity. N = 7/group; *P < 0.05 vs. WKY; #P < 0.05 vs. SHR.

Fig. 3. Representative western blots of (A) eNOS (150 kDa), nNOS-α (160 kDa), and nNOS-β (140 kDa) in WKY rats and SHRs at age 10 weeks. Relative abundance of renal cortical (B) eNOS, (C) nNOS-α, and (D) nNOS-β as quantified. N = 8/group; A10 = SHRs treated with aliskiren 10 mg/kg/day; A30 = SHRs treated with aliskiren 30 mg/kg/day; *P < 0.05 vs. WKY; #P < 0.05 vs. SHR; §P < 0.05 A10 vs. A30.

Table 1 Plasma and tissue L-arginine and ADMA levels in WKY rats and SHRs at age 10 weeks

	WKY	SHR	SHR+A10	SHR+A30
Plasma (μM)				
L-arginine	87.7 ± 5.1	79.5 ± 3.3	73.5 ± 1.2 [a]	78.9 ± 2.9
ADMA	0.53 ± 0.03	0.8 ± 0.05[a]	0.67 ± 0.02[a,b]	0.64 ± 0.02 [a,b]
L-arginine-to-ADMA ratio	168 ± 11	102 ± 7 [a]	110 ± 3 [a]	124 ± 5 [a,b,c]
Kidney (μM/mg protein)				
L-arginine	44.2 ± 2.0	40.2 ± 2.9	47.6 ± 2.5	44.0 ± 2.2
ADMA	1.1 ± 0.08	1.34 ± 0.09	1.25 ± 0.1	0.99 ± 0.03[b,c]
L-arginine-to-ADMA ratio	42 ± 4	32 ± 4	40 ± 4	45 ± 3[b]

SHR+A10 = SHR+aliskiren 10 mg/kg/day; SHR+A30 = SHR+aliskiren 30 mg/kg/day; N = 8/group; [a]P < 0.05 vs. WKY; [b]P < 0.05 vs. SHR; [c]P < 0.05 SHR+A10 vs. SHR+A30.

***Response to Reviewers**

Dear Dr. Nijkamp,

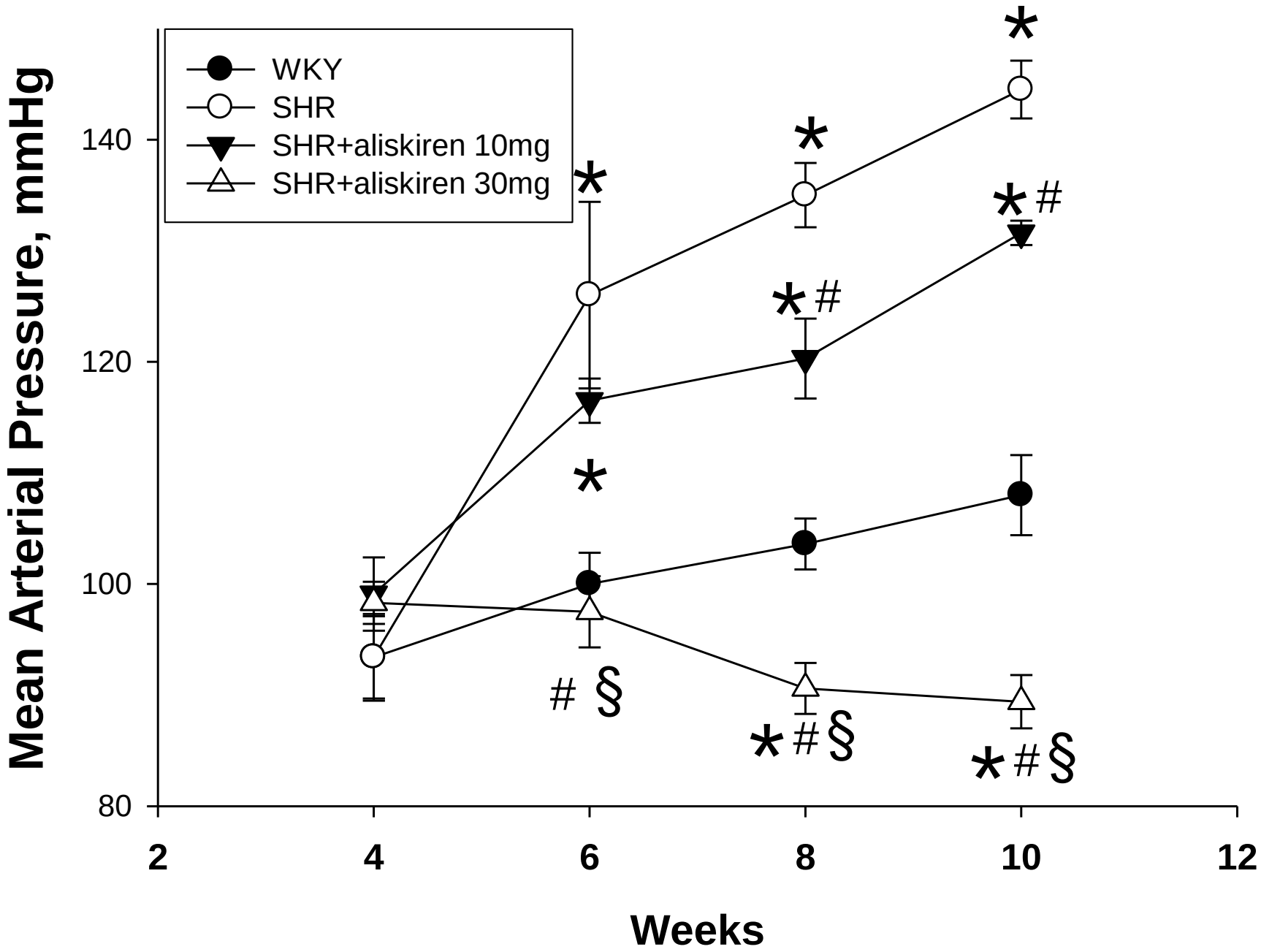
Thank you very much for your letter regarding our article ID EJP-34522R2 entitled "Aliskiren prevents hypertension and reduces asymmetric dimethylarginine in young spontaneously hypertensive rats". We greatly thank for your kindness to give us opportunity for publication.

We have change AT1 receptor into angiotensin AT_1 receptor in the manuscript that you recommended. Thank you very much for your review and publication in your journal.

Yours Sincerely,

You-Lin Tain, MD, PhD
Division of Pediatric Nephrology
Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan
E-mail: tainyl@hotmail.com or tainyl@adm.cgmh.com.tw
Office phone: +886-7-7317123 ext. 8723
Phone: +886-0975-056995
Fax: +886-7-7338009
Mailing address: Department of Pediatrics, Kaohsiung Chang Gung Memorial Hospital, 123, Dabi Rd., Niausung, Kaohsiung, 833 Taiwan

Figure 1


WKY
SHR
SHR+aliskiren 10mg
SHR+aliskiren 30mg
Mean Arterial Pressure, mmHg
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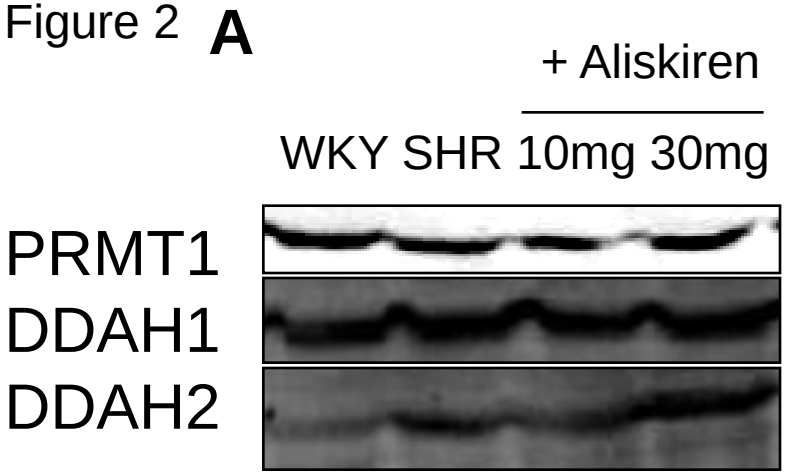
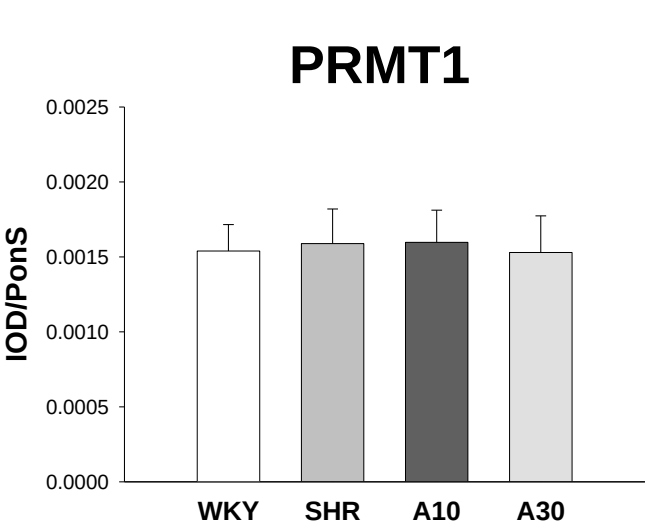
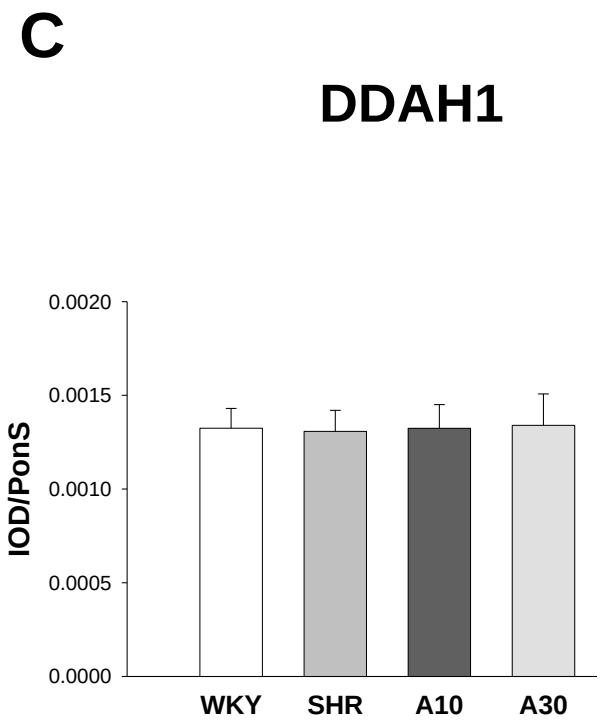
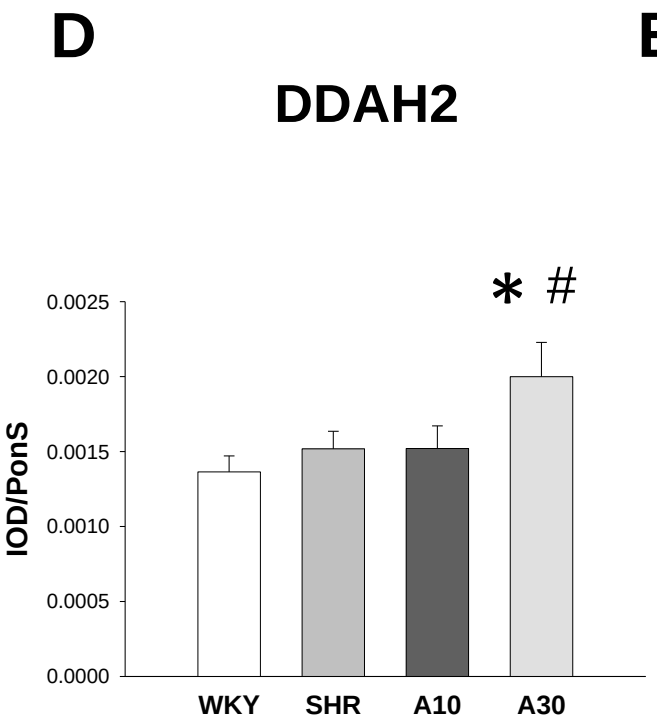
*#§

Figure 2


A
+ Aliskiren
WKY SHR 10mg 30mg
PRMT1
DDAH1
DDAH2


B
PRMT1
IOD/PonS
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
WKY
SHR
A10
A30


C
DDAH1
IOD/PonS
0.0020
0.0015
0.0010
0.0005
0.0000
WKY
SHR
A10
A30


D
DDAH2
* #
IOD/PonS
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
WKY
SHR
A10
A30

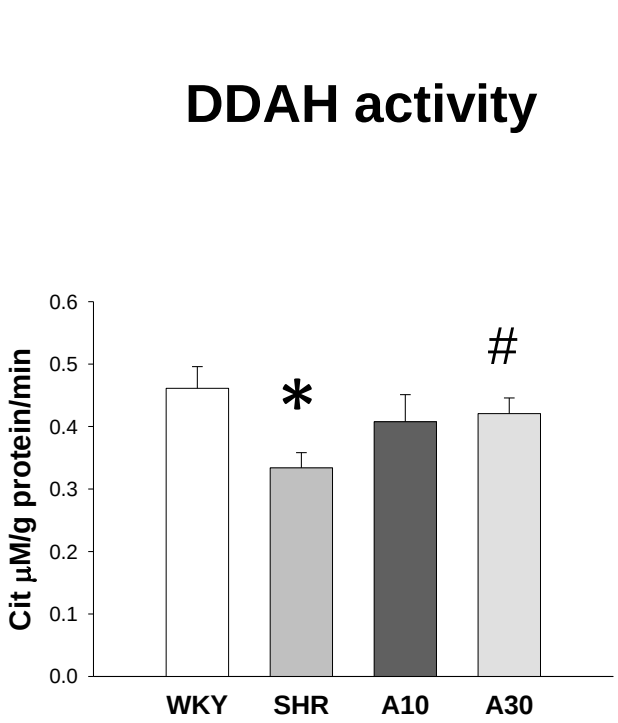
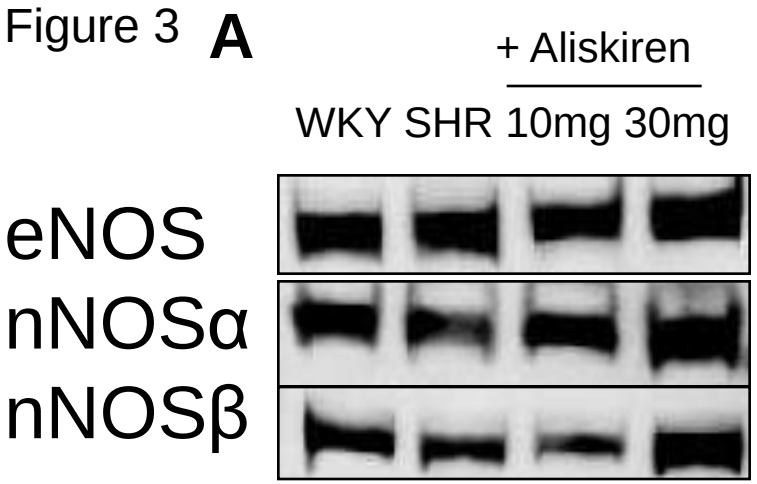
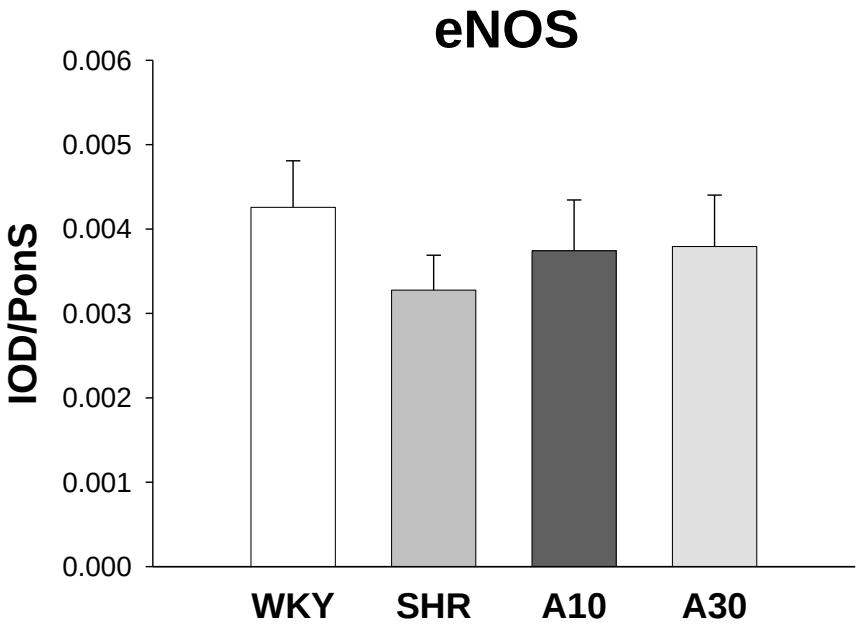
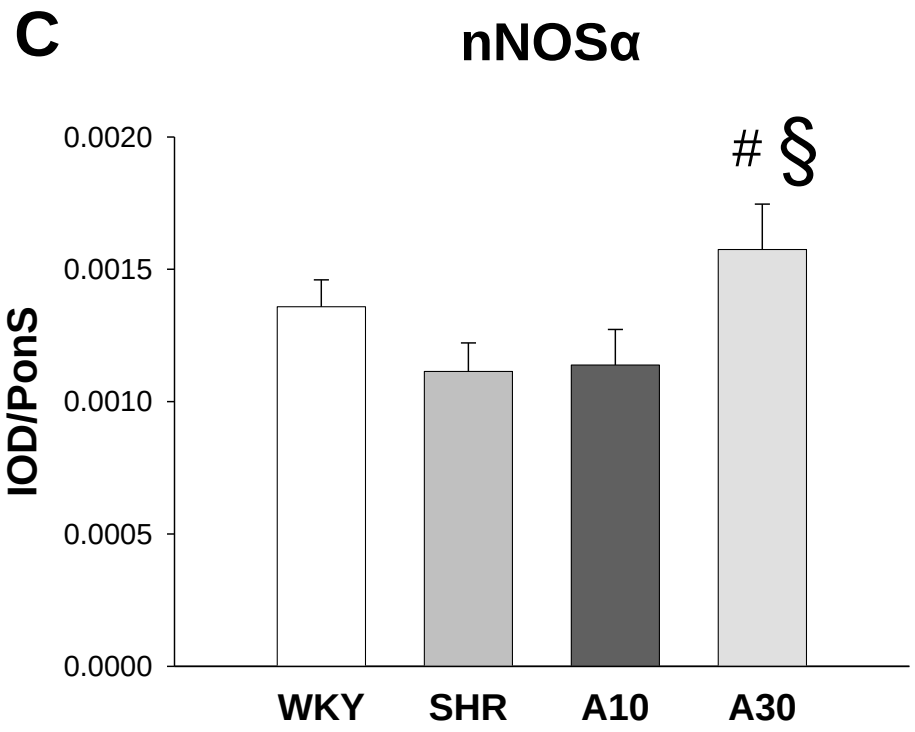
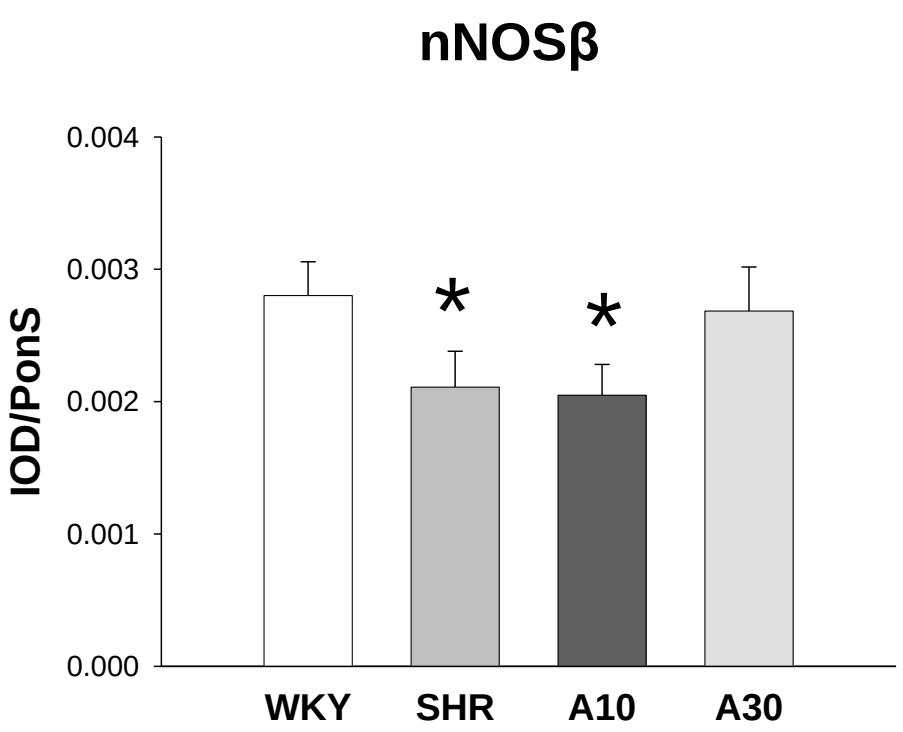

E
DDAH activity
Cit μM/g protein/min
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
#
WKY
SHR
A10
A30

Figure 3

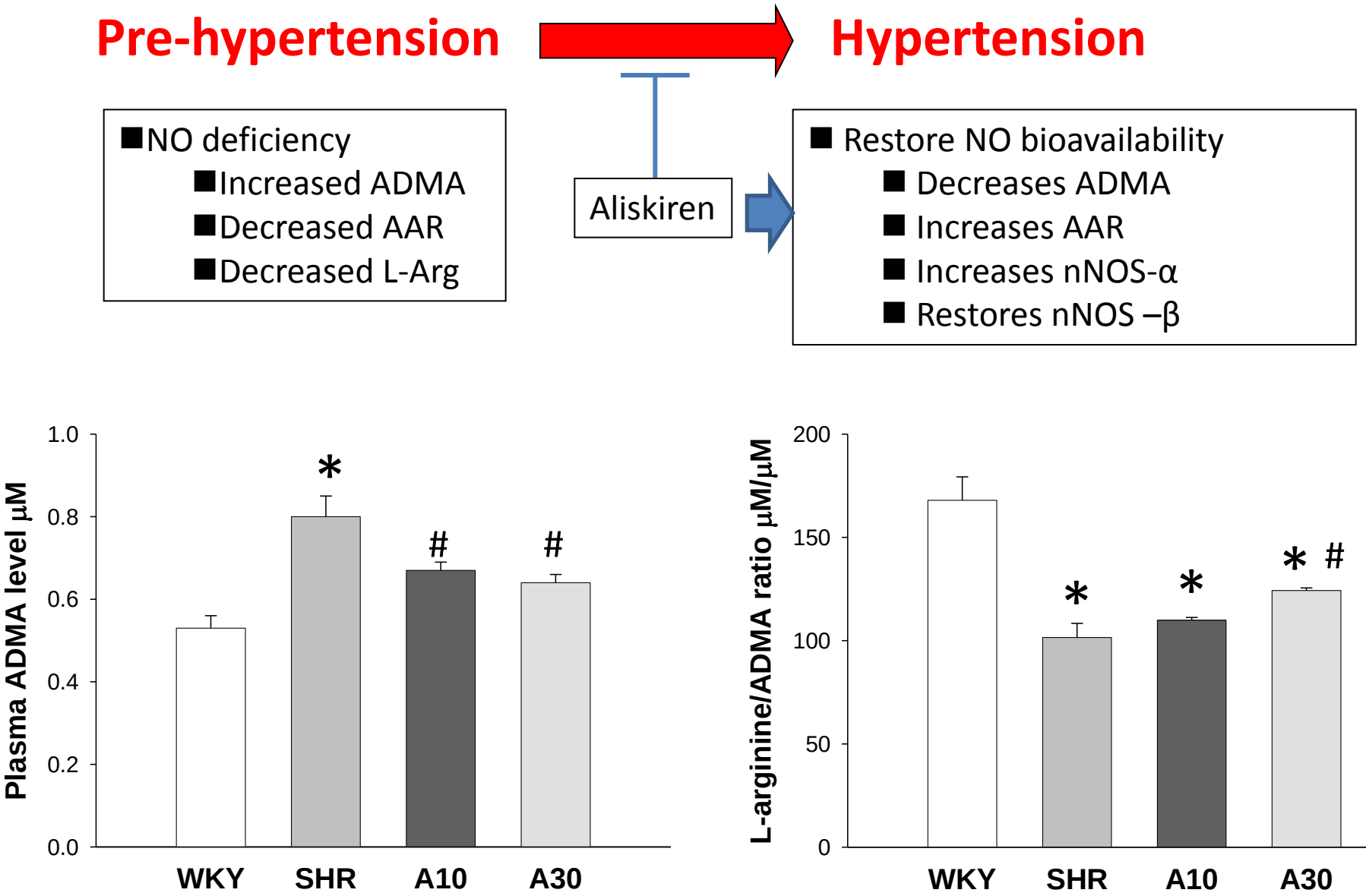

A
+ Aliskiren
WKY SHR 10mg 30mg
eNOS
nNOSα
nNOSβ


B
eNOS
IOD/PonS
0.006
0.005
0.004
0.003
0.002
0.001
0.000
WKY
SHR
A10
A30


C
nNOSα
IOD/PonS
0.0020
0.0015
0.0010
0.0005
0.0000
§
WKY
SHR
A10
A30


D
nNOSβ
IOD/PonS
0.004
0.003
0.002
0.001
0.000
*
*
WKY
SHR
A10
A30

Graphical Abstract



*P < 0.05 vs. WKY; #P < 0.05 vs. SHR; A10 = SHR+aliskiren 10 mg/kg/day; A30 = SHR+ aliskiren 30 mg/kg/day