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Differential gene expression in the kidneys of SHR and WKY rats after intravenous administration of *Akkermansia muciniphila*-derived extracellular vesicles

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Although *Akkermansia muciniphila* (Am) plays a beneficial role as a probiotic in the treatment of metabolic syndrome, the mechanisms remain elusive. We tested the hypothesis that Am extracellular vesicles (AmEVs) protect against hypertension through modulation of gene expression in the kidneys of spontaneously hypertensive rats (SHRs). Extracellular vesicles purified from anaerobically cultured Am (1.0×10^8 or 1.0×10^9 particles/kg) or vehicles were injected into the tail veins of Wistar-Kyoto rats (WKYs) and SHRs weekly for 4 weeks. Renal cortical tissues isolated from both rat strains were analyzed by trichrome stain and RT-qPCR. AmEVs protect against the development of hypertension in SHRs without a serious adverse reaction. AmEVs increased the expression of vasocontracting *Agt* and *At1ar* as well as vasodilating *At2r*, *Mas1* and *Nos2* in the kidneys of both strains. These results indicate that AmEVs have a protective effect against hypertension without a serious adverse reaction. Therefore, it is foreseen that AmEVs may be utilized as a novel therapeutic for the treatment of hypertension.

Keywords Akkermansia muciniphila, Hypertension, Extracellular vesicles, Differential Gene Expression, Spontaneous hypertensive rats

Akkermansia muciniphila (Am), belonging to the phylum Verrucomicrobiota, was discovered in 2002. This mucin-degrading bacterium features an oval shape, with a cell size ranging from 0.6 to 1.0 µm. It is Gram-negative, thrives under obligate anaerobic conditions, and lacks motility[1]. Typically found in the mammalian intestinal microbiota community, Am has not only been reported to enhance host metabolism and reduce inflammation but has also been considered a health marker when present in human fecal samples[2,3]. Am is currently a subject of therapeutic research for various metabolic-associated diseases[4] since, like many other gut microbes, they synthesize several metabolites. Microbiota-derived metabolites are either beneficial (for example, short-chain fatty acids and indole-3-lactic acid) or detrimental (for example, trimethylamine N-oxide). They can activate several downstream signaling pathways via G-protein-coupled receptors or through direct immune cell activation. These alterations activate mechanisms that are traditionally associated with blood pressure regulation, such as the renin–angiotensin–aldosterone system, the autonomic nervous system, and the immune system[5].

Extracellular vesicles (EVs), small membranous nanoparticles derived from various cell types, including gut microbes, have emerged as crucial mediators in cell-to-cell communication, influencing a wide range of physiological and pathological processes, particularly in the realm of immunity[6,7]. Microbiota-derived EVs may exhibit several bacterial cell surface components, including outer membrane proteins, lipids, lipopolysaccharides, and peptidoglycans, they can carry various bacterial components such as DNA, RNAs, enzymes, toxins,

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and a complex of microbial-associated molecular patterns[8]. Administering EVs from specific bacterial strains such as *Escherichia coli* is highlighted for its potential to modulate immune signalling pathways, host nutrition, and bacterial metabolite production, emphasizing their role in immunomodulation and signalling pathways[9].

The renin–angiotensin–aldosterone system (RAAS) is an important regulator of blood pressure that plays a crucial role in essential hypertension. Despite extensive research, the precise etiology of this condition remains elusive, with contributing mechanisms encompassing factors such as immune system activation, heightened sympathetic nervous system activity, dysregulation of water and salt homeostasis, and alterations in gut microbiota composition[10–14]. Components of the RAAS pathway not only influence renal sodium and water balance, vascular tone, and sympathetic output but also directly modulate immune cell activation and function[15]. The kidney, a primary target for various RAAS components, houses two distinct branches: the classic ACE/AngII/AT1R/aldosterone axis and the non-classical ACE2/Ang 1–7/MasR axis. The latter, characterized by ACE2/Ang 1–7/MasR and Ang 1–9, counteracts the effects of the classic arm, serving as a crucial player in preventing inflammation, oxidative stress, hypertension, and cardiovascular remodeling. Recognizing the significance of this counter-regulatory arm underscores its potential as a therapeutic target for managing cardiovascular comorbidities[16,17].

Despite the growing evidence linking gut microbiota dysbiosis with hypertension, and the increasing use of probiotics[18–21], there remains a gap in understanding the direct application of gut bacteria-derived EVs on blood pressure and its regulatory systems. Building upon our prior study demonstrating the anti-inflammatory and anti-hypertensive effects of intravenously administered Am-derived EVs (AmEVs), this study aims to investigate AmEVs modulation of the renal RAAS and regulation of blood pressure. We tested the hypothesis that Am extracellular vesicles (AmEVs) protect against hypertension through modulation of gene expression in the kidneys of spontaneously hypertensive rats (SHRs).

Results

Intravenous administration of AmEVs prevents an increase in blood pressure in SHR recipients

Systolic blood pressure (SBP) was measured using the non-invasive tail-cuff method in SHRs and WKYs before (Fig. 1A) and after (Fig. 1B) four weeks of intravenous administration of AmEVs. The animals were injected with either a vehicle or two different dosages of AmEVs: 1.0×10^8 or 1.0×10^9 particles/kg. SBP significantly decreased in SHRs treated with 1.0×10^9 particles/kg of AmEVs compared to the vehicle group (Fig. 1B). However, histological analysis of SHRs and WKYs kidneys revealed no significant differences in tissue structure and fibrotic areas after intravenous administration of AmEVs at dosages of 1.0×10^8 or 1.0×10^9 particles/kg (Fig. 2B).

AmEVs increase the mRNA expression of renal RAAS components in SHRs and WKYs

To determine whether intravenous administration of AmEVs affects the mRNA expression of renal renin–angiotensin–aldosterone system (RAAS) components in both SHRs and WKYs. In response to AmEVs injection, an upregulation of angiotensinogen (*Agt*) mRNA expression was observed in both SHRs and WKYs (Fig. 3A). Concurrently, renin (*Ren*) expression exhibited a notable increase exclusively in SHRs (Fig. 3B), emphasizing a differential response to AmEVs in hypertensive conditions. In contrast, pro-renin receptor (*Atp6ap2*) expression showed no significant change (Fig. 3C).

AmEVs intervention also triggered a significant upregulation of angiotensin-converting enzyme 1 (*Ace1*) mRNA expression in both SHRs and WKYs (Fig. 4A). Notably, *Ace2* expression demonstrated a substantial rise, particularly in WKYs (Fig. 4B) subjected to the higher AmEVs dosage, further highlighting the nuanced effects of

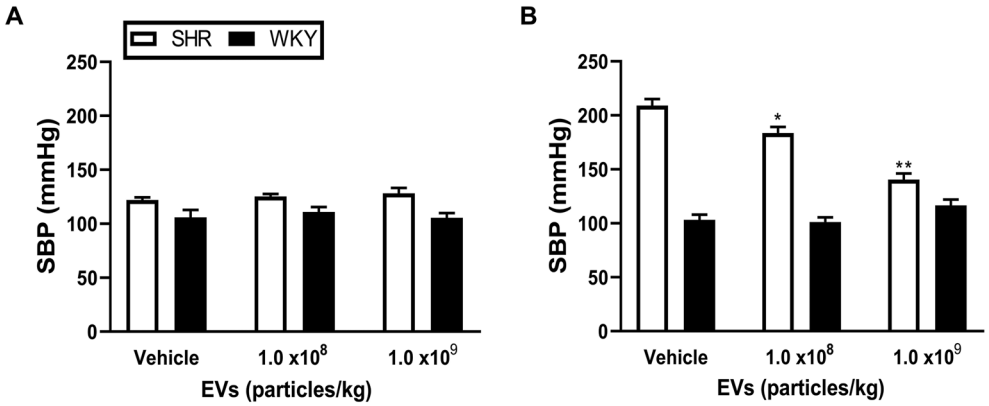

Figure 1. Effects of AmEVs on the systolic Blood Pressure (SBP) in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKYs). SBP was measured using the tail-cuff method. (**A**) Baseline SBP readings before the treatment commenced. (**B**) SBP readings after intravenous administration of AmEVs for four weeks. Animals were randomly grouped and injected once a week with either a vehicle or two different dosages of AmEVs: 1.0×10^8 or 1.0×10^9 particles/kg. Treatment with 1.0×10^9particles/kg of AmEVs significantly reduced SBP in SHRs. Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was performed using a two-way analysis of variance followed by Tukey's post-hoc multiple comparisons test. $^*p < 0.05$ and $^{**}p < 0.01$ vs. SHR vehicle.

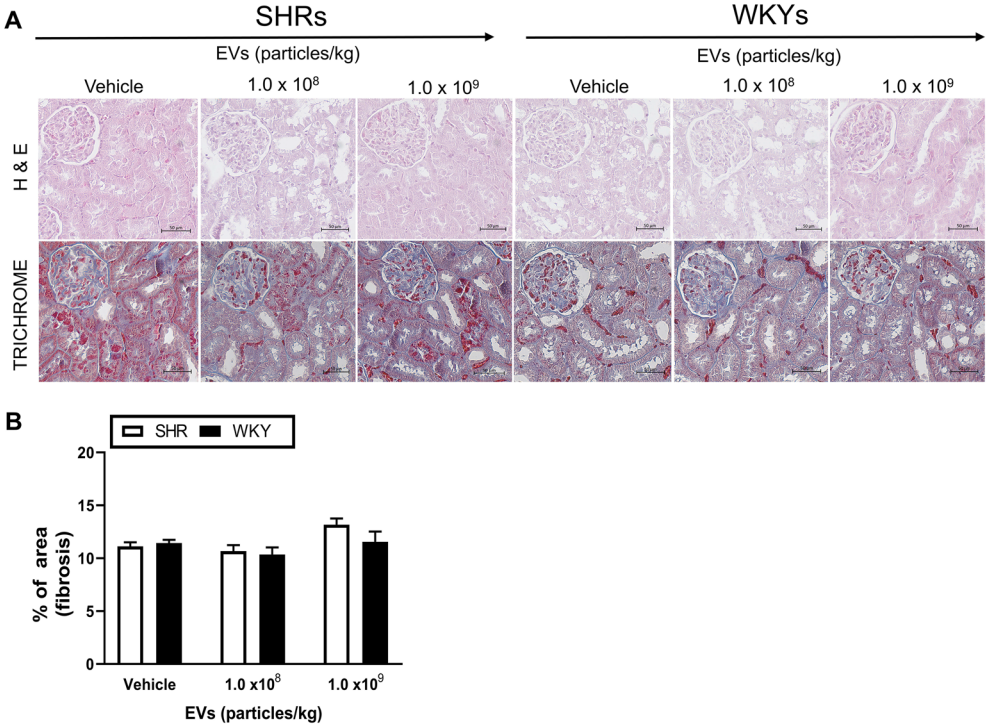

Figure 2. Histological analysis of SHRs and WKYs kidneys following the intravenous administration of AmEVs. Representative microscopic images of kidneys from SHRs and WKYs injected with a vehicle or two different dosages of AmEVs (1.0×10^8 or 1.0×10^9 particles/kg). Kidney sections were stained with hematoxylin and eosin (H & E) or Masson's trichrome (Trichrome). Scale bars for 100 × magnification are 50 µm (**A**). The fibrotic area was quantified using ImageJ software, and no significant differences in tissue fibrosis were observed, as presented in graph (**B**). Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was performed using a two-way analysis of variance followed by Tukey's post-hoc multiple comparisons test.

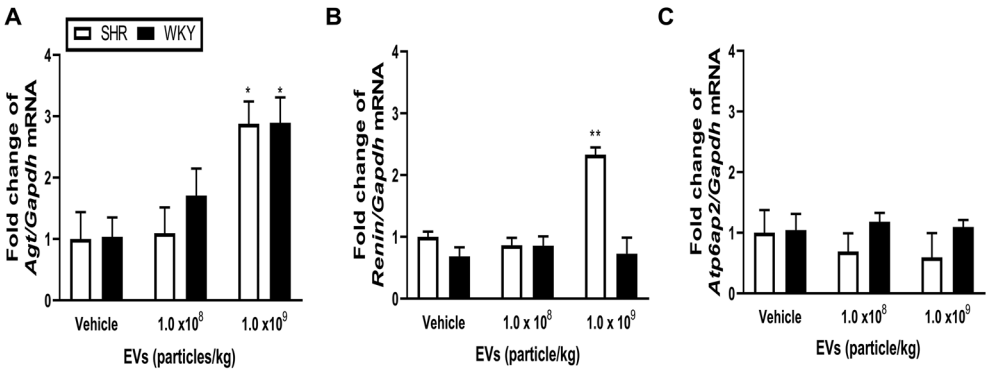

Figure 3. Effect of AmEVs on the mRNA expression of angiotensinogen, pro-renin and renin in the kidney of SHR and WKY rats. The mRNA expression of *Agt*, *Renin and Atp6ap2* was assessed by RT-qPCR in the kidneys of SHRs and WKYs injected with a vehicle or two different dosages of AmEVs (1.0×10^8 or 1.0×10^9 particles/kg). AmEVs treatment significantly increased the expression of angiotensinogen in both SHRs and WKYs injected with 1.0×10^9 particles/kg (**A**) while renin expression increased only in SHRs administered with 1.0×10^9 particles/kg (**B**). However, it did not affect pro-renin receptor expression levels (**C**). Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was conducted using a two-way analysis of variance followed by Tukey's post hoc multiple comparisons test. $^*p < 0.05$ and $^{**}p < 0.01$ vs. SHR vehicle.

AmEVs on the RAAS components. Examining the downstream receptors, mRNA expression levels of angiotensin II type 1 receptor a (*At1ar*) and angiotensin II type 2 receptor (*At2r*) exhibited a significant increase in both SHRs and WKYs that were injected with 1.0×10^9 particles/kg of AmEVs (Fig. 5A and B), indicating a comprehensive impact on angiotensin receptor expression. Additionally, Mas receptor (*Mas*) expression significantly increases in SHRs and WKYs with the same AmEVs dosage (Fig. 5C).

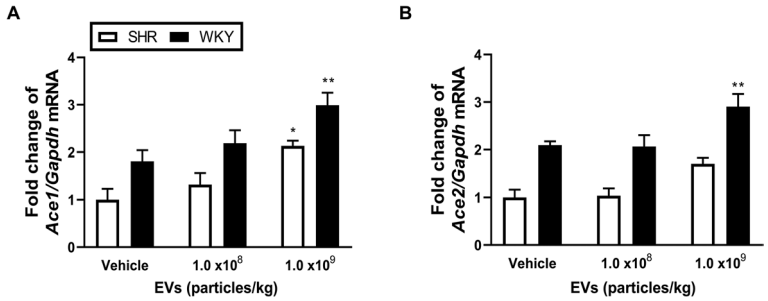

Figure 4. Effect of AmEVs on the mRNA expression of both angiotensin-converting enzyme (ACE) I and II in SHR and WKY rats. The mRNA expression levels of *Ace1* and *Ace2* were evaluated using RT-qPCR in the kidneys of SHRs and WKYs following treatment with AmEVs. Animals were administered a vehicle or two different dosages of AmEVs (1.0×10^8 or 1.0×10^9 particles/kg). AmEVs injection resulted in a significant increase in *Ace1* expression in both SHRs and WKYs injected with 1.0×10^9 particles/kg (**A**). *Ace2* expression was significantly elevated only in WKYs administered with the higher dosage (**B**). Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was conducted using a two-way analysis of variance followed by Tukey's post hoc multiple comparisons test. $^*p < 0.05$ and $^{**}p < 0.01$ vs. SHR vehicle.

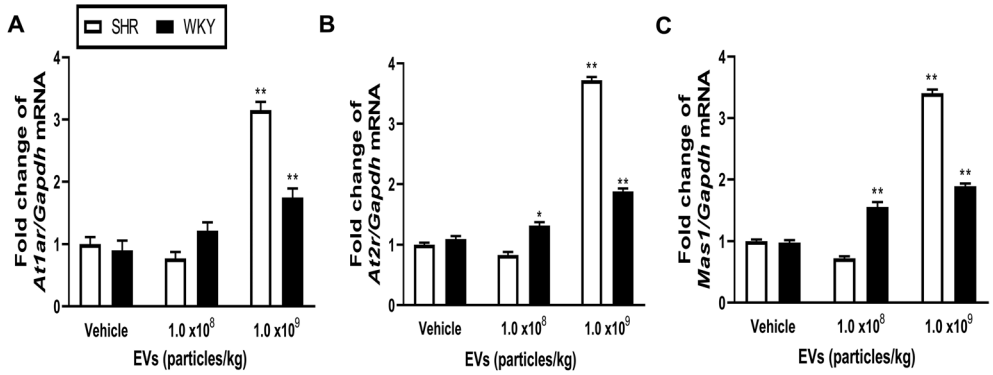

Figure 5. Effect of AmEVs on the mRNA expression of RAAS-associated receptors in both SHR and WKY rats. The mRNA expression levels of *AT1aR*, *AT2R* and *Mas1* were examined using RT-qPCR in the kidneys of SHRs and WKYs following treatment with AmEVs. Animals were administered a vehicle or two different dosages of AmEVs (1.0×10^8 or 1.0×10^9 particles/kg). AmEVs injection resulted in a significant increase in both *AT1aR*, *AT2R* and *Mas1* expression in SHRs and WKYs injected with 1.0×10^9 particles/kg (**A**–**C**). A moderate elevation of *AT2R* and *Mas1* expression was observed in WKYs administered with 1.0×10^8 particles/kg (**B** and **C**). Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was conducted using a two-way analysis of variance followed by Tukey's post-hoc multiple comparisons test. $^*p < 0.05$ and $^{**}p < 0.01$ vs. SHR vehicle.

Intravenous administration of AmEVs increases the mRNA expression of ROS-producing enzymes in SHRs and WKYs

To determine whether the administration of AmEVs affects the mRNA expression of NADPH oxidases (*Nox1, Nox2,* and *Nox4*) in both SHRs and WKYs. AmEVs injection elicited a substantial increase in the mRNA expression levels of *Nox1* and *Nox2*, indicative of heightened oxidative stress, in both SHRs and WKYs subjected to the higher dosage of 1.0×10^9 particles/kg (Fig. 6B and C). Concurrently, inducible nitric oxide synthase (*Nos2*) expression also significantly increased, further accentuating the impact of AmEVs on the oxidative stress pathways (Fig. 6A). Interestingly, a modest elevation in *Nox4* expression was observed (Fig. 6D), suggesting a nuanced response in the regulation of this specific NADPH oxidase.

Contrastingly, AmEVs exhibited no significant effect on the mRNA expression levels of the antioxidant enzymes superoxide dismutase 1 and 2 (*Sod1* and *Sod2*), as well as catalase (*Cat*), across all experimental groups encompassing both SHRs and WKYs (Fig. 7A–C).

Discussion

We demonstrate that Am extracellular vesicles (AmEVs) protect against hypertension through modulation of gene expression in the kidneys of spontaneously hypertensive rats (SHRs). Our findings show that administering AmEVs at a dose of 1.0×10^9 particles/kg resulted in an increase in the expression of *Agt, Ace1, At1ar, At2r,* and *Mas1* in both SHRs and WKYs. We observed a significant rise in *Renin* expression only in SHRs and a marked increase in *Ace2* exclusively in WKYs. The expression of the pro-renin receptor (*Atp6ap2*) remained unchanged.

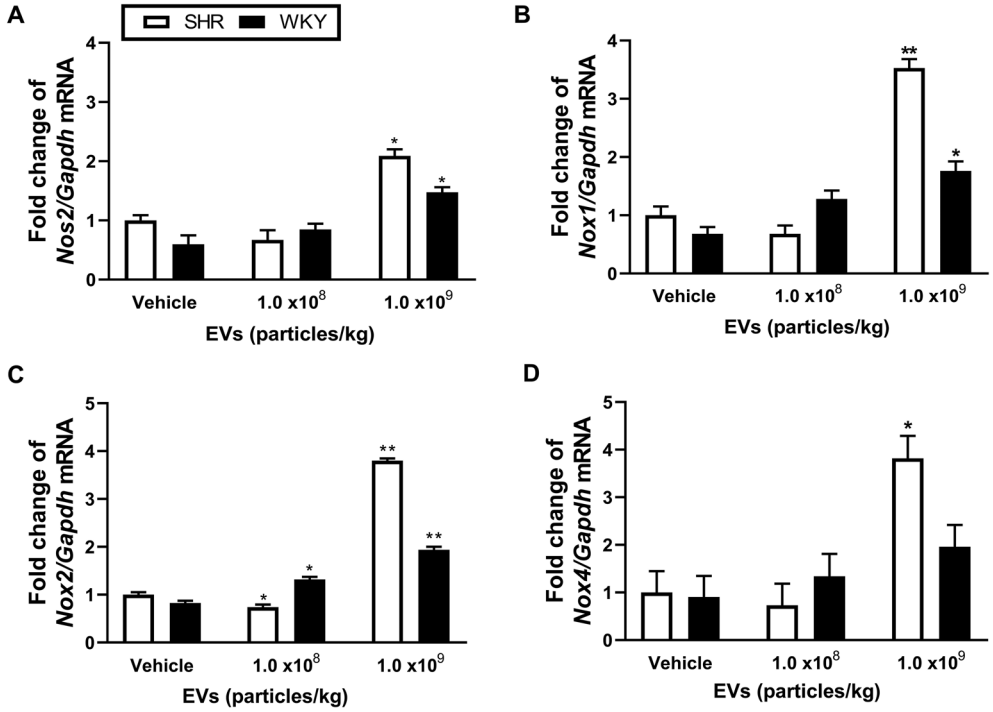

Figure 6. Effect of AmEVs on the mRNA expression of reactive oxygen species (ROS) producing enzymes in SHR and WKY rats. The mRNA expression levels of NADPH oxidases (NOX1, 2, and 4) and inducible nitric oxide synthase (iNOS), were assessed using RT-qPCR in the kidneys of SHRs and WKYs following treatment with AmEVs. Animals were administered a vehicle or two different dosages of AmEVs (1.0×10^8 or 1.0×10^9 particles/kg). AmEVs injection resulted in a significant increase in *Nox 1* (**B**,**C**) and 2, and *Nos2* (**A**) in both SHRs and WKYs injected with 1.0×10^9 particles/kg, with a slight increase in *Nox4* (**D**) expression levels. Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was conducted using a two-way analysis of variance followed by Tukey's post-hoc multiple comparisons test. $^*p < 0.05$ and $^{**}p < 0.01$ vs. SHR vehicle.

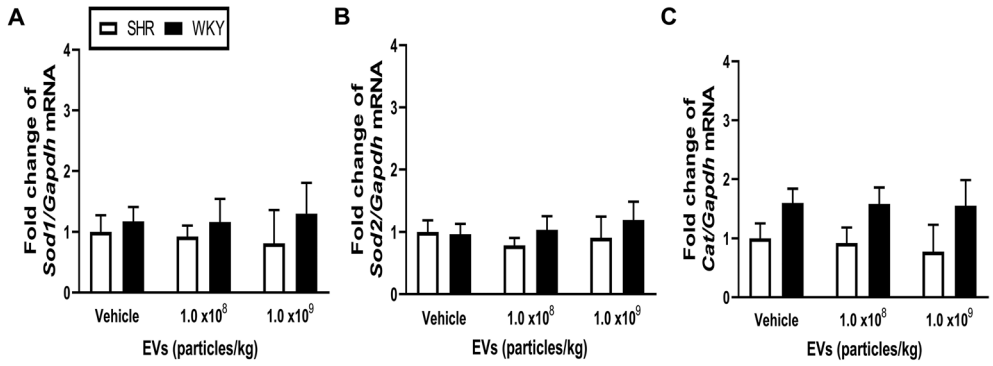

Figure 7. Effect of AmEVs on the mRNA expression of antioxidant enzymes in SHR and WKY rats. The mRNA expression levels of superoxide dismutase 1 (*Sod1*), superoxide dismutase 2 (*Sod2*), and catalase (*Cat*) (**A**–**C**) were examined using RT-qPCR in the kidneys of SHRs and WKYs following treatment with AmEVs. Animals were administered a vehicle or two different dosages of AmEVs (1.0×10^8 or 1.0×10^9 particles/kg). AmEVs had no significant effect on these enzymes in both SHRs and WKYs across all treatment groups. Data are presented as mean ± SEM, with n = 6 animals per group. Statistical analysis was conducted using a two-way analysis of variance followed by Tukey's post-hoc multiple comparisons test.

Intravenous administration of 1.0×10^9particles/kg AmEVs demonstrated a notable antihypertensive effect by suppressing the rise in systolic blood pressure (SBP) in SHRs compared to the vehicle group (Fig. 1B). Histological analysis of kidney tissues revealed no significant differences in structure and fibrotic areas, indicating the absence of adverse effects on renal morphology (Fig. 2). However, the administration of AmEVs (1.0×10^9 particles/kg) led to an increase in the mRNA expression of *Ace1* in both SHRs and WKYs, whereas *Ace2* showed

a significant increase only in WKYs. In SHRs injected with the same AmEVs dosage, there was an observed increase compared to the vehicle group, although the differences were not statistically significant (Fig. 4). The observed increase in *Ace1* expression in both SHRs and WKYs may be attributed to the response in ligands and receptors, especially in normotensive rats, aimed at maintaining stable blood pressure. ACE, primarily known for cleaving Ang I to Ang II, also cleaves various substrates, including bradykinin[22]. Increased *Ace2* mRNA expression holds potential benefits for hypertension by promoting the generation of vasodilatory and anti-inflammatory peptides, as the enzyme cleaves Ang I into the inactive Ang 1–9, further converted by ACE into the Ang 1–7[23]. Besides that, ACE2 also cleaves Ang II into Ang 1–7 which acts on the G-coupled protein Mas receptor to counteract the effects of Ang II in blood pressure regulation[24]. Previous studies have indicated that hypertension in SHRs is associated with an increase in *Ace1* mRNA expression, while *Agt*, *Renin*, and *At1ar* remain relatively constant when compared to normotensive WKYs[25]. Furthermore, RAAS-related gene expression in 16-week-old SHRs and WKYs demonstrated elevated expression in *Ace1, Ace2, Agt, At1ar*, and *Atp6ap2* in SHRs compared to WKYs while *Mas1* and *Renin* expression between the animal types remain the same[26].

AmEVs (1.0×10^9 particles/kg) intervention resulted in a significant increase in mRNA expression levels of both *At1ar* and *At2r* in both SHRs and WKYs, highlighting an effect on angiotensin receptor expression (Fig. 5A and B). Activation of AT1aR by Ang II induces elevated intracellular calcium levels, generates reactive oxygen species (ROS), and triggers vasoconstriction, proliferation, and hypertrophy of vascular smooth cells[27,28]. In contrast, Ang II activation of AT2R exerts a counter-regulatory effect against Ang II/AT1R activation. Concurrently, Mas receptor (*Mas1*) expression significantly increased in response to AmEVs (1.0×10^9 particles/kg), emphasizing nuanced effects on RAAS components (Fig. 5C). Mas1, a G-protein-coupled receptor similar to AT2R, mediates the effects of Ang 1–7[29], acting primarily in opposition to the vasoconstrictive effects of Ang II through AT1R. Furthermore, Ang 1–7 has been reported to be antithrombotic, and anti-proliferative[30,31]. Previous research indicates that the ACE2/Ang 1–7/Mas axis has protective effects beyond cardiovascular outcomes, especially in inflammatory control. Under normal conditions, AT2R is expressed at low levels, but their expression significantly rises under pathological conditions such as hypertension, cardiac hypertrophy, and heart failure, where their actions predominate over those of AT1R[32–34]. The concurrent high expression of *At1ar*, *At2r*, and *Mas1* in SHRs and WKYs injected with AmEVs (1.0×10^9 particles/kg) suggests that AmEVs may have a cardioprotective effect.

The significant upregulation of angiotensinogen mRNA expression in both SHRs and WKYs upon administration of AmEVs 1.0×10^9 particles/kg underscores a pivotal modulation of this key RAAS precursor. However, the mRNA expression of *Renin* showed a significant increase only in SHRs (Fig. 3A and B). In contrast, the pro-renin receptor (*Atp6ap2*) showed no significant difference across all groups, suggesting a subtle change in response to AmEVs in SHRs (Fig. 3C). Previous studies have demonstrated that the use of ACE inhibitors or angiotensin receptor blockers can elevate the mRNA expression of *Agt* and *Renin* in hypertensive rats, attributed to a compensatory mechanism[35–37]. These suggest that further investigation is needed to reveal the exact mechanism involved in this modulation, indicating that the observed genetic modification in renal RAAS activation may involve a more complex pathway.

Interestingly, our study found that both SHRs and WKYs administered with 1.0×10^9 particles/kg AmEVs showed a significant increase in the expression of oxidative stress-related genes, in addition to the responses observed in the RAAS components. Specifically, *Nox1*, *Nox2*, *Nox4*, and iNOS were substantially upregulated (Fig. 6). Some studies have reported that Am can decrease reactive oxygen species (ROS) production[38]. However, conflicting studies reported that, as a gram-negative bacterium, Am activated lipopolysaccharides (LPS) in CKD mice, leading to NADPH oxidase-dependent-ROS production[39]. The abundance of Am may degrade more mucus in the gut, which could damage the mucosal barrier and cause the leak of inflammatory markers, resulting in inflammation and the high production of ROS, particularly Nox1 and iNOS[40,41]. It is worth noting that Nox4 may have a distinct role, releasing hydrogen peroxide (H_2O_2) which can induce vasorelaxation unlike Nox1, Nox2, Nox3 and Nox5, which release superoxide[42].

However, the mRNA expression of antioxidant-related genes, *Sod1 and Sod2* and *Cat*, showed no significant differences (Fig. 7). Previous research reported that the activities of SOD, CAT, and GPX were unaffected in the cortex and medulla of untreated SHRs despite indications of oxidative stress[43]. In an Am study where the supplement was orally administered, it enriched gut-produced bacteria and interacted with them to produce positive effects[44]. As EVs typically contain genetic materials from their parent cells, it is plausible that AmEVs carry microbial DNA that could induce NADPH oxidases[45]. However, the lack of tissue damage observed in our study indicates that the increased expression of NADPH oxidase did not lead to overt inflammation, outlining the need for further investigation into the long-term effects of AmEVs administration. It is also important to note that, while gene expression analysis provides valuable information about transcriptional changes within cells; it does not directly measure protein levels or activity, which are critical determinants of biological function.

In conclusion, our study provides valuable insights into the complex interplay of molecular pathways underlying the antihypertensive effects of AmEVs in SHRs and WKYs. While the observed changes in RAAS components and oxidative stress-related genes warrant further investigation, our findings highlight the potential therapeutic implications of AmEVs in hypertension management. Future studies exploring the long-term effects of AmEVs administration and their impact on cardiovascular outcomes will be crucial for elucidating the full therapeutic potential of AmEVs in hypertension and the mechanism behind their modulation of gene expression.

Materials and methods

Am culture and EVs isolation

Bacteria culture and EVs isolation as previously described by Kim et al. Am was obtained from the KCTC Korean Collection for Type Cultures in Daejeon, South Korea. The bacteria were cultured in a mucin-based medium at

37 °C until the optical density reached 1.5 at 600 nm. The cultures were then centrifuged at 6000 g for 20 min, and the supernatants were filtered using a 0.22 μm pore filter. The filtrate was pelleted by ultracentrifugation in a 45 Ti rotor at 100,000 × g for 2 h at 4 °C. The final pellets were resuspended in phosphate-buffered saline (PBS) and stored at − 80 °C. The extracted EVs were assessed using transmission electron microscopy (TEM) and protein band patterns were compared between bacterial pellets and EVs using SDS-PAGE with silver staining[46].

Animals

Tissue samples for this study were sourced from a prior experiment[46]. These samples were obtained post-anesthetization via intraperitoneal injection of 50 mg/kg sodium pentobarbital, stored at -80 °C, and thawed at room temperature for the current study. In vivo experiments conducted were approved by the Kyungpook National University Clinical Review Board (approval number 2022–0456), and all procedures for animal experiments described in this study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Emphasis was placed on minimizing animal usage and reducing suffering Four-week-old male spontaneous hypertensive rats (SHRs) and Wistar Kyoto rats (WKYs) were procured from Japan SLC (Hamamatsu, Shizuoka, Japan). After two weeks of acclimatization, rats received weekly doses of either vehicle (PBS, Gibco), 1.0×10^8, or 1.0×10^9 particles/kg of *Akkermansia muciniphila* extracellular vesicles (AmEVs) for 4 weeks. Random assignment was ensured, and the rats had ad libitum access to food (SAFE® D40; Paris, France) and water.

Blood pressure measurement

The systolic blood pressure (SBP) was assessed using the non-invasive tail-cuff method. Rats underwent a preheating phase on a hotplate at 35 °C for 10 min and were subsequently positioned in plastic restrainers. A cuff, equipped with a pneumatic pulse sensor, was secured around the tail. The CODA system (Kent Scientific Corporation, Torrington, CT, USA) recorded blood pressure values while heating. The average blood pressure was calculated from a minimum of ten consecutive readings per rat (Supplementary information).

Histological analysis

The kidneys were fixed in 4% formalin, dehydrated, and embedded in paraffin using conventional methods. Paraffin-embedded samples were sectioned to a thickness of 3 μm. Sections were stained with hematoxylin and eosin (H&E, BBC Biochemical, McKinney, TX, USA) and Masson's trichrome (BBC Biochemical). After staining, slides were examined using light microscopy. The area of kidney fibrosis was measured using ImageJ software (National Institute of Health, Bethesda, MD, U.S.A).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Tissues (approximately 100 mg) were homogenized in liquid nitrogen with a glass homogenizer. Total RNA was extracted using QIAzol® Lysis Reagent (QIAGEN Science, Germantown, MD, USA) following the manufacturer's instructions. The extracted RNA was then converted into cDNA using RevertAid™ First-Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions. RT-qPCR was then performed using a QuantStudio 5 real-time PCR instrument (Thermo Fisher Scientific). The reaction solution (10 μL) comprised 5 μL of SYBR Green master mix (New England Biolabs, Ipswich, MA, USA), 2 μL of water, 2 μL of cDNA, and 1 μL of primer set (200 nmol/L). The RT-qPCR was conducted as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles at 95 °C for 15 s, followed by 1 min at 60 °C. $2^{-\Delta\Delta Ct}$ was calculated using GAPDH as a reference gene to determine relative mRNA expression levels. Primer sets used in this study are presented in Table 1.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Graph Pad Prism 8 (GraphPad Software, San Diego, CA, U.S.A.) with a *p-value* of < 0.05 considered significant.

Genes for rat (accession no.)	Primer sequence (5' -3')
AGT (NM_134432)	F: AGAACCCCAGTGTGGAGACG R: AGCCAACCTTTGAGCCTGTGCCCA
RENIN (NM_012642)	F: GTAACTGTGGGTGGAATCATTGTG R: TGGGAGAGAATGTGGTCGAAGA
ACE1 (NM_12544)	F: CGGGTCGCAGAGGAATTCTT R: CCTGAAGTCCTTCCTGTTGTAGA
ACE2 (NM_001012006.2)	F: TCAGAGCTGGGATGCAGAAA R: GGCTCAGTCAGCATGGAGTTT
AT1Ra (NM_030985.4)	F: GGAGAGGATTCGTGGCTTGAG R: CTTTCTGGGAGGGTTGTGTGAT
AT2R (NM_012494.3)	F: CATCACCAGCAGTCTTCCTTTTG R: AAAACAGTGAGACCACAACAATGT
MAS1 (XM_039101421.1)	F: ACTGCCGGGCGGTCATCATC R: GGTGGAGAAAAGCAAGGAGA
ATP6AP2 (NM_001007091.1)	F: GGTGACAGTGAAGGGGGTA R: GGGCCAACTGCAAAACTACA
NOS2 (XM_039085203.1)	F: GGACCACCTCTATCAGGAA R: CCTCATGATAACGTTTCTGGC
NOX1 (NM_053683.2)	F: AATTGGTCTCCCAAAGGAGGT R: CAGGTAGAGAACAAGGTCCC
NOX2 (NM_023965.1)	F: GCATTCACACACCACTCCAC R: TCCTTCCTCTCCTAAGGC
NOX4 (NM_053524.1)	F: AAAACCCTCCAGGCAAAGAT R: TCGTCGTCGTCGTACATCTT
SOD1 (NM_017050.1)	F: GCAGGGCGTCATTCACTT R: AGACTCAGACCACATAGGGA
SOD2 (NM_017051.2)	F: AGCTAGGCTTCCTGACTGACA R: AGGCCCTGCATACTTTGTCC
CAT (NM_012520.2)	F: GAGACAGTGTACTGCAAGTTCC R: GGGACAGTTCACAGGTATCTGC
GAPDH (NM_001394060.2)	F: ATGACTCTACCCACGGCAAG R: CTGGAAGATGGTGATGGGTT

Table 1. Primer sequences for RT-qPCR.

Data availability

The data supporting these findings is available upon request from the corresponding author, inkim@knu.ac.kr.

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O.Z.Y.: methodology, formal analysis, investigation, data curation, writing the original draft, reviewing and editing, and visualization. C.W.K.: technical expertise, data analysis, writing—review and editing, project administration. J.Y.K.: methodology, investigation, formal analysis. S.M.J.: Investigation and Writing—Review and Editing. K.I.K.: conceptualization, validation, resources, writing—review and editing, supervision, funding acquisition. All authors carefully read, revised, and approved the article for submission.

Competing interests

The authors declare no competing interests.

Additional information

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