

## **Streptococcal Exotoxin Streptolysin O Causes Vascular Endothelial Dysfunction Through PKC $\beta$ Activation**

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**Running Title:** Streptolysin O-impaired vascular endothelial function

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**List of nonstandard abbreviations**

acetylcholine: ACh

endothelial NO synthase: eNOS

enzyme-linked immunosorbent assay: ELISA

Glyceraldehyde phosphate dehydrogenase: GAPDH

immunoglobulin G: IgG

L-N<sup>G</sup>-nitro arginine methyl ester: L-NAME

lipopolysaccharide: LPS

mitogen-activated protein kinase: MAPK

polyvinylidene fluoride: PVDF

protein kinase C: PKC

sodium dodecyl sulfate polyacrylamide gel electrophoresis: SDS-PAGE

spontaneous hypertensive rats: SHRs

*Streptococcus dysgalactiae* subsp. *equisimilis*: SDSE

streptolysin O: SLO

Wistar Kyoto rats: WKYs

**Section:** Cardiovascular

## Abstract

Streptolysin O (SLO) is produced by common hemolytic streptococci that cause a wide range of diseases from pharyngitis to life-threatening necrotizing fasciitis and toxic shock syndrome. While the importance of SLO in invasive hemolytic streptococcus infection has been well demonstrated, the role of circulating SLO in non-invasive infection remains unclear. The aim of this study was to characterize the pharmacological effect of SLO on vascular functions, focusing on cellular signaling pathways. In control Wistar rats, SLO treatment (1–1000 ng/mL) impaired acetylcholine-induced endothelial-dependent relaxation in the aorta and second-order mesenteric artery in a dose-dependent manner, without any effects on sodium nitroprusside-induced endothelium-independent relaxation or agonist-induced contractions. SLO also increased phosphorylation of the endothelial NO synthase (eNOS) inhibitory site at Thr495 in the aorta. Pharmacological analysis indicated that either endothelial dysfunction or eNOS phosphorylation was mediated by protein kinase C $\beta$  (PKC $\beta$ ), but not by the p38 mitogen-activated protein kinase (MAPK) pathway. Consistent with this, SLO increased phosphorylation levels of PKC substrates in the aorta. *In vivo* study of control Wistar rats indicated that intravenous administration of SLO did not change basal blood pressure, but significantly counteracted the acetylcholine-induced decrease in blood pressure. Interestingly, plasma anti-SLO IgG levels were significantly higher in 10- to 15-week-old spontaneously hypertensive rats compared to age-matched control rats ( $P < 0.05$ ). These findings demonstrated that SLO causes vascular endothelial dysfunction, which is mediated by PKC $\beta$ -induced phosphorylation of the eNOS inhibitory site.

### **Significance Statement**

This study showed for the first time, that *in vitro* exposure of vascular tissues to SLO impairs endothelial function, an effect that is mediated by PKC $\beta$ -induced phosphorylation of the eNOS inhibitory site. Intravenous administration of SLO in control and hypertensive rats blunted the ACh-induced decrease in blood pressure, providing evidence for a possible role of SLO in dysregulation of blood pressure.

**Keywords:** streptolysin O, endothelium, protein kinase C $\beta$ , hypertension

## Introduction

Members of the genus *Streptococcus* include several important human and animal pathogens (Barnett et al., 2015). One of the most clinically significant human pathogens is *Streptococcus pyogenes* (Group A streptococci), which annually causes 700 million infections worldwide (Carapetis et al., 2005) and is responsible for a wide range of diseases that is mediated by the production of several extracellular toxins (Bolz et al., 2015; Hancz et al., 2019). Group A streptococci are known colonizers of the oropharynx, genital mucosa, rectum, and skin in healthy adults and children (Efstratiou and Lamagni, 2016). *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) (Group C and G streptococci) is microbiologically similar to *S. pyogenes*, and constitutes an emerging human pathogen causing diseases ranging from simple pharyngitis to life-threatening toxic shock syndrome (Baracco, 2019). Group C and G streptococci are widely distributed in both human and animals and are colonizers of the skin, pharynx, gastrointestinal tract, and genital tract (Efstratiou, 1997).

Among the exotoxins produced by hemolytic streptococci, streptolysin O (SLO) is the major virulence factor, and is considered to contribute to necrotizing fasciitis and toxic shock syndrome, conditions that include cardiomyocyte contractile dysfunction (Bolz et al., 2015), hyper-stimulation of mast cells (Stassen et al., 2003), impairment of neutrophil oxidative burst (Uchiyama et al., 2015), and bacterial growth (Lu et al., 2015). While the importance of SLO in invasive hemolytic streptococcus infection has been well demonstrated, SLO can be detected at low levels in plasma under standard physiological conditions or apparent non-invasive infection (Kotby et al., 2012); indeed, low-level SLO may affect local or systemic circulation. However, the role of low-level SLO in circulation remains unclear at present.

The present study sought to analyze the possible effects of SLO on vascular endothelial and smooth muscle functions. This study showed, for the first time (to our knowledge), that *in vitro* exposure of vascular tissues to SLO impairs endothelial function, an effect that is mediated by protein kinase C (PKC)  $\beta$ -induced phosphorylation of the endothelial NO synthase (eNOS) inhibitory site. Interestingly, intravenous administration of SLO in control and hypertensive rats blunted the acetylcholine (ACh)-induced decrease in blood pressure, providing evidence for a possible role of SLO in cardiovascular disorders.

## Methods

### *Animals*

Experimental animals used in this study were male Wistar rats (7–14 weeks old), spontaneous hypertensive rats (SHRs) (10–15 weeks old), and Wistar Kyoto rats (WKYs) (10–15 weeks old), which served as controls for SHRs. Care of these animals met standards set forth by the National Institutes of Health guidelines for the care and use of experimental animals. All procedures were approved by the Animal Care and Use Committee of the Okayama University of Science.

### *Blood pressure measurement using the tail-cuff method*

Systolic blood pressure in WKYs and SHRs was measured using the tail-cuff method (Softron, Tokyo, Japan) as previously described (Mukohda *et al.*, 2020). Rats were trained to reduce stress before starting measurements, and blood pressure was measured at room temperature without a heater.

### *Plasma concentration of anti-SLO immunoglobulin G (IgG)*

Plasma was collected from WKYs and SHRs. Anti-SLO IgG levels then were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Abbkine, Wuhan, China).

### *Vascular function*

Aortic and mesenteric arterial functions were assessed using a wire myograph



preparation as previously described (Mukohda et al., 2019). The thoracic aortas or secondary branches of mesenteric arteries were carefully dissected and cut into small pieces. The vascular preparations were suspended in an organ bath containing Krebs buffer (mmol/L: 118.3 NaCl, 4.7 KCl, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, and 11 glucose) maintained at 37 °C and 95% O<sub>2</sub>/5% CO<sub>2</sub>. Aortas or mesenteric arteries then were equilibrated for 45 min under a resting tension of 0.5 or 0.03–0.05 g, and contraction was recorded in response to KCl (100 mmol/L).

Concentration-dependent response curves to phenylephrine (1 nmol/L–30 μmol/L), serotonin (10 nmol/L–30 μmol/L), and angiotensin II (0.1 nmol/L–3 μmol/L) were performed. In addition, concentration-dependent response curves to ACh (0.3 nmol/L–30 μmol/L) or sodium nitroprusside (SNP; 0.1 nmol/L–30 μmol/L) were performed after an initial submaximal precontraction (60–80%) with phenylephrine (30–300 nmol/L) for aortas or U46619 (a thromboxane A<sub>2</sub> receptor agonist; 1–10 μmol/L) for mesenteric arteries.

### *Western blotting*

Thoracic aortas were cleaned of perivascular fat and snap-frozen in liquid nitrogen. Frozen samples then were homogenized in lysis buffer containing 50 mmol/L Tris·Cl buffer, 0.1 mmol/L EDTA (pH 7.5), 1% (m/vol) Na deoxycholic acid, 1% (vol/vol) Nonidet P-40, and 0.1% (vol/vol) sodium dodecyl sulfate (SDS) with protease inhibitor and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan). Tissues were subjected to rotary shaking for 1 hr at 4 °C and then centrifuged (20,000 g) for 10 min at 4 °C. The protein concentration in the resulting supernatant was determined by a Lowry assay (Nacalai Tesque). Equal amounts of proteins (10–20 μg) were separated by

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8–12%) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, MA, USA). Membranes were blocked with 5% skim milk and incubated with primary antibodies at 4 °C overnight; bands then were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h). Antibodies against eNOS (BD Biosciences, San Jose, CA, USA), phospho-eNOS Thr495 (BD Biosciences), p38 mitogen-activated protein kinase (MAPK; Proteintech), phospho-p38 MAPK (Cell Signaling Technology, MA, USA), and phospho-PKC substrate (Cell Signaling Technology, MA, USA) were used for these experiments. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control; the corresponding antibody was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

#### *Direct blood pressure measurement*

After Wistar rat or SHR was anesthetized with isoflurane, catheters were inserted into the common carotid artery for direct blood pressure measurement and into the femoral vein for drug administration. Blood pressure was measured by BP transducer (Nihon Kohden) and recorded by BP Amp (Nihon Kohden) and PowerLab system (ADInstruments). Concentration-dependent response curves to SLO (0.64–640 ng/kg [equivalence 0.01–10 ng/mL]) or ACh (0.02–20 µg/kg [equivalence 1 nmol/L–1 µmol/L]) were performed after stable blood pressure was achieved. In some experiments, animals were pretreated for 15 min with SLO, or vehicle.

### *Chemicals*

SLO, angiotensin II, ACh, SNP, human serum albumin and PKC inhibitor Ro31-8220 were purchased from Sigma-Aldrich (St. Louis, MO). Phenylephrine, serotonin, U46619, and L-N<sup>G</sup>-nitro arginine methyl ester (L-NAME; a nonselective inhibitor of NOS) were obtained from Wako (Tokyo, Japan). We also used SB203580, a p38 MAPK inhibitor purchased from Adipogen (San Diego, CA, USA); LY333531, a PKC $\beta$  inhibitor purchased from abcam (Cambridge, UK); and CGP53353, a selective PKC $\beta$ 2 inhibitor purchased from Tocris Bioscience (Bristol, UK).

### *Statistical analysis*

Experiments were performed in similar numbers between control and treated groups. Results are expressed as mean $\pm$ SEM. Statistical evaluation of the data was performed using Prism (GraphPad San Diego, CA, USA). Where appropriate, a two-tailed paired or unpaired Student's t-test was used to compare data between two groups. In other experiments, one- or two-way ANOVA (repeated measures when appropriate) with *post hoc* Tukey's tests was used to compare data between more than two groups. Differences were considered significant at  $P < 0.05$ . Note that, in cumulative concentration-response curves, the asterisk regards entire curve as significant.

## Results

### *SLO does not affect vascular smooth muscle contractile activity*

Initially, we examined the *in vitro* effect of SLO (1–100 ng/mL, 30 min) on vascular smooth muscle contractile activity in aortas isolated from control Wistar rats. SLO did not change contractile responses to phenylephrine (Figure 1A), serotonin (Figure 1B), or angiotensin II (Figure 1C). Higher concentration of SLO (300 and 1000 ng/ml) also did not change contractile responses to phenylephrine (Figure 1D). These results indicate that SLO used at concentrations of 1–1000 ng/mL does not affect vascular smooth muscle contractile activity.

### *SLO impairs endothelium-dependent vasodilation and eNOS phosphorylation*

Next, we investigated whether SLO affects endothelium-dependent vasodilation in aortas isolated from Wistar rats. SLO treatment (10–1000 ng/mL) for 30 min blunted ACh-induced endothelium-dependent relaxation in a dose-dependent manner (Figure 2A). Notably, 1000 ng/ml SLO severely decreased the ACh-induced relaxation. In contrast, SLO (1–1000 ng/mL) did not affect SNP-induced endothelial-independent relaxation (Figure 2B). We also examined the effect of SLO on second-order mesenteric artery and found that lower SLO concentrations (1–10 ng/mL) impaired ACh-induced relaxation (Figure 2C). There was no difference in SNP-induced relaxation in SLO-treated mesenteric arteries (Figure 2D). These data indicated that SLO causes endothelial dysfunction without affecting contractile responses in arterial vessels. It also was noted that the mesenteric artery (peripheral artery) seemed more sensitive to SLO than did the aorta (elastic artery).

It has been reported that human serum albumin can bind to SLO with high affinity and neutralizes the cytotoxic effects of SLO (Vita et al., 2020). Therefore, we next investigated the effect of SLO (100 ng/ml) in presence of 1% human serum albumin *in vitro*. Although albumin (1%) seemed to partially suppress the SLO-mediated impairment of ACh-induced relaxation, SLO still significantly impaired the relaxation (Figure 2E).

ACh-induced vasodilation largely depends on eNOS activity, which can be evaluated by the eNOS phosphorylation level (Chen et al., 1999). In aortas isolated from Wistar rats, SLO increased the level of eNOS phosphorylated at Thr495, a modification that inhibits enzymatic activity (Figure 2F and 2G). Total eNOS expression was not changed by SLO treatment, indicating that SLO-induced impairment of ACh relaxation is due to dysfunctional eNOS activity.

#### *Endothelial dysfunction is mediated by PKC $\beta$ , but not by p38 MAPK*

SLO activates the p38 MAPK and PKC pathways in mast cells (Chen et al., 1999). We examined whether p38 MAPK is involved in the endothelial dysfunction caused by SLO. SB203850 (10  $\mu$ mol/L, 30 min), a p38 MAPK inhibitor, did not change SLO-mediated impairment of ACh-induced relaxation in aortas from Wistar rats (Figure 3A). However, SLO (100 ng/mL, 30 min) increased p38 MAPK phosphorylation (Figure 3B and 3C).

Next, we examined whether PKC activation plays some role in SLO-induced endothelial dysfunction. Ro31-8220 (100 nmol/L, 30 min), a pan-PKC inhibitor, restored ACh-mediated dilation in the presence of SLO (100 ng/mL) in aortas from

Wistar rats (Figure 3D). Impaired ACh-induced relaxation also was restored by LY333531 (1  $\mu\text{mol/L}$ , 30 min), a PKC $\beta$  inhibitor (Figure 3E). Furthermore, CGP53353 (10  $\mu\text{mol/L}$ , 30 min), a selective PKC $\beta$ 2 inhibitor, restored ACh-induced relaxation in the presence of SLO (Figure 3F). SLO-induced eNOS phosphorylation at Thr495 was reversed by pretreatment with Ro31-8220 (100 nmol/L, 30 min) or CGP53353 (10  $\mu\text{mol/L}$ , 30 min) (Figure 3G and 3H). Consistent with these findings, SLO significantly increased phosphorylation levels of PKC substrates in the aorta (Figure 3I and 3J). Taken together, these results indicated that SLO impairs vascular endothelial function via activation of PKC $\beta$ , but not via the p38 MAPK pathway.

#### *SLO impairs blood pressure regulation in Wistar rat*

We next examined the effect of SLO on blood pressure *in vivo*. SLO was administered intravenously in Wistar rats and blood pressure was measured by direct monitoring of arterial pressure. Administration of SLO (0.64–640 ng/kg) did not change basal mean blood pressure, heart rate, or pulse pressure (Figure 4A–4C). However, ACh (0.02–20  $\mu\text{g/kg}$ ) decreased blood pressure and heart rate. Prior administration of SLO (640 ng/kg, 15 min) significantly blunted ACh-induced blood pressure (Figure 4D). SLO treatment also blunted ACh (20  $\mu\text{g/kg}$ ) -induced heart rate reduction (Figure 4E).

#### *Plasma anti-SLO IgG is elevated in SHRs*

It has been reported that hypertension is associated with increased intestinal permeability (Santisteban *et al.*, 2017), which may be secondary to increases in

plasma levels of lipopolysaccharide (LPS, also termed endotoxin) (Toral et al., 2019). We therefore examined whether the plasma SLO level is elevated in hypertension by measuring the levels of anti-SLO IgG in 10- to 15-week-old SHR. We preliminarily confirmed that systolic blood pressure and heart weight were increased in SHR compared to age-matched control WKYs (Figure 5A and 5B). The concentration of anti-SLO IgG also was significantly elevated in SHR compared to age-matched WKYs ( $P < 0.05$ ) (Figure 5C), indicating that plasma levels of SLO are elevated in SHR compared to WKYs.

#### *SLO impairs blood pressure regulation in SHR*

We finally examined the effect of SLO on blood pressure in SHR. Administration of SLO (0.64–640 ng/kg) in SHR did not change basal mean blood pressure, heart rate, or pulse pressure (Figure 6A–6C). Administration of SLO (640 ng/kg, 15 min) significantly blunted ACh-induced blood pressure (Figure 6D). SLO treatment also blunted the ACh (20  $\mu$ g/kg) -induced heart rate reduction (Figure 6E).

## Discussion

Streptolysin O (SLO) is produced by common hemolytic streptococci including Group A streptococci and Group C and G streptococci causing diseases ranging from simple pharyngitis to life-threatening toxic shock syndrome. The importance of SLO in invasive hemolytic streptococcus infection has been reported, however the role of circulating low-level SLO in non-invasive infection remains unclear. We examined the pharmacological effect of SLO on vascular functions, focusing on cellular signaling pathways.

### *Change in vascular functions and cell signaling*

Treatment of rat vascular tissues with SLO impaired ACh-induced endothelial-dependent relaxation in a dose-dependent manner, with no changes in vascular smooth muscle contractile activities. Impaired endothelial function was accompanied by increased phosphorylation at the eNOS inhibitor site at Thr495. SLO-induced endothelial dysfunction and decreased eNOS phosphorylation were attenuated by pan-PKC inhibitor and PKC $\beta$ -selective inhibitors. Treatment of the aorta with SLO also increased p38 MAPK phosphorylation; however, a p38 MAPK inhibitor did not restore endothelial dysfunction. These results suggested that the PKC $\beta$  pathway, but not the p38 pathway, contributes to SLO-induced vascular dysfunction.

SLO induces inflammatory cytokine production by activating the p38 MAPK and PKC pathways in mast cells (Stassen *et al.*, 2003) and the NF- $\kappa$ B pathway in monocytes and epithelial cells (Dragneva *et al.*, 2001). PKC activity is related to several cardiovascular diseases, and several reports have addressed the importance of



PKC $\beta$  activity in this context (Inoguchi *et al.*, 1992; Koya *et al.*, 1997; Ohshiro *et al.*, 2006; Meier *et al.*, 2007; Mehta *et al.*, 2009; Chiasson *et al.*, 2011; Tabit *et al.*, 2013). Our study demonstrated that SLO induces PKC activation, and PKC $\beta$ -selective inhibitors restore SLO-induced endothelial dysfunction via abrogation of inhibitory eNOS phosphorylation (Thr495).

Recently, Vita *et al.* reported that serum albumin protects cells from cytotoxic effects and cell membrane permeabilization induced by SLO (Vita *et al.*, 2020), suggesting the presence of physiologically important buffering system for this toxin. However, our results showed that the impairment of ACh-induced endothelial-dependent relaxation due to SLO was scarcely affected in presence of albumin (Figure 2E). Previous reports have suggested that SLO mediates changes in cell signaling via both pore-forming action (ionophore-like action) and pore-forming-independent pathways in a concentration-dependent manner (Stassen *et al.*, 2003; Magassa *et al.*, 2010; Logsdon *et al.*, 2011; Kano *et al.*, 2012; Uchiyama *et al.*, 2015). Because the concentration of SLO (1–100 ng/mL) used in our study was relatively lower than the concentration necessary to induce the pore-dependent pathway, we speculate that some specific mechanism exists in cells to recognize low concentration of SLO. This pathway seems to be resisted to the albumin-mediated protect system.

### *Blood pressure regulation*

In this study, we also examined the effect of SLO administration on cardiovascular responses *in vivo*. Although SLO did not affect basal mean blood pressure, heart rate, or pulse pressure in Wistar rats as well as SHR, this molecule significantly counteracted the ACh-induced blood pressure reduction and heart rate reduction.

These results obtained in *in vivo* study reasonably explain the results of *in vitro* study using isolated vascular tissues.

*Pathophysiological significance evaluated in a hypertension model*

It has been reported that endotoxin (LPS) can be detected in systemic circulation at low concentrations (<200 pg/mL) in healthy subjects (Wiedermann *et al.*, 1999; Freudenberg *et al.*, 2008). Plasma endotoxin concentrations were higher in patients with edematous heart failure than in patients without edematous heart failure (Niebauer *et al.*, 1999). In animal models, endotoxin derived from the gut microbiome has been hypothesized to promote weight gain and diabetes (Cani *et al.*, 2007) and to exacerbate atherosclerotic lesions (Li *et al.*, 2016).

Due to technical limitation, the precise plasma levels of SLO in healthy humans and also in patients infected with the streptococcal infection remain unclear. Group A streptococcal infection can be clinically diagnosed by post-streptococcal antibodies, including anti-SLO and anti-deoxyribonuclease B antibodies (Kaplan *et al.*, 1998). Under healthy conditions, circulating anti-SLO IgG is detected at concentrations of  $\leq 200$  IU/mL (Kotby *et al.*, 2012). SLO also is produced by many strains of Group C and G streptococci, which are common normal microbiota in both human and animals, where these bacteria serve as colonizers of the skin, pharynx, gastrointestinal tract, and genital tract (Efstratiou, 1997). A cohort study showed that the level of post-streptococcal antibodies is increased in metabolic syndrome patients (Aran *et al.*, 2011). In the present study, we analyzed the plasma levels of anti-SLO IgG in the SHR hypertension model, and found that the anti-SLO IgG level was significantly elevated in SHRs compared to age-matched WKYs. In this study, although we found

an acute blunting effect of SLO on endothelium-dependent relaxation, we failed to observe any changes in the basal blood pressure. Further study is needed to clarify the role of SLO in the blood pressure regulation.

Recently, several reports have revealed that gut dysbiosis is linked to cardiovascular diseases, including hypertension, both in human clinical studies (Li et al., 2017) and in animal models (Yang et al., 2015). Elevation of plasma endotoxin has been reported in a hypertensive animal model, accompanied by increased intestinal permeability (Toral et al., 2019). Our study indicated that the level of streptococcal exotoxin is increased in a hypertensive animal model; this elevation of exotoxin concentration may cause vascular endothelial dysfunction. Because an increase in vascular endothelial permeability may be closely associated with a disruption of the intestinal barrier in inflammation, further study will be necessary to elucidate the potential effects of SLO on the endothelial cells of the intestinal vascular wall. Future studies of the methodological exploration analyzing precise plasma level and tissue accumulation of SLO are also warranted to fully define role of circulating SLO.

### *Conclusion*

Increased intestinal permeability has been suggested as an important risk factor for inflammation, including cardiovascular diseases, and endothelial dysfunction is key for cardiovascular disease associated with inflammation. In the present study, we demonstrated that the streptococcal exotoxin SLO causes vascular endothelial dysfunction, which is mediated by PKC $\beta$ -induced phosphorylation of the eNOS inhibitory site. This finding is expected to contribute to further understanding of the potential role(s) of circulating enteric toxin in the pathogenesis of cardiovascular

diseases including hypertension.

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*Participated in research design:* Mukohda and Ozaki

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*Wrote or contributed to the writing of the manuscript:* Mukohda, Nakamura, Takeya, Matsuda, Yano, Mizuno and Ozaki

## References

- Aran A, Lin L, Finn LA, Weiner K, Peppard P, Young T and Mignot E (2011) Post-streptococcal antibodies are associated with metabolic syndrome in a population-based cohort. *PLoS One* **6**:e25017.
- Baracco GJ (2019) Infections Caused by Group C and G Streptococcus (Streptococcus dysgalactiae subsp. equisimilis and Others): Epidemiological and Clinical Aspects. *Microbiol Spectr* **7**.
- Barnett TC, Cole JN, Rivera-Hernandez T, Henningham A, Paton JC, Nizet V and Walker MJ (2015) Streptococcal toxins: role in pathogenesis and disease. *Cell Microbiol* **17**:1721-1741.
- Bolz DD, Li Z, McIndoo ER, Tweten RK, Bryant AE and Stevens DL (2015) Cardiac myocyte dysfunction induced by streptolysin O is membrane pore and calcium dependent. *Shock* **43**:178-184.
- Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC and Burcelin R (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**:1761-1772.
- Carapetis JR, Steer AC, Mulholland EK and Weber M (2005) The global burden of group A streptococcal diseases. *Lancet Infect Dis* **5**:685-694.

Chen ZP, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR and Kemp BE (1999) AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett* **443**:285-289.

Chiasson VL, Quinn MA, Young KJ and Mitchell BM (2011) Protein kinase C $\beta$ -mediated phosphorylation of endothelial nitric oxide synthase threonine 495 mediates the endothelial dysfunction induced by FK506 (tacrolimus). *J Pharmacol Exp Ther* **337**:718-723.

Dragneva Y, Anuradha CD, Valeva A, Hoffmann A, Bhakdi S and Husmann M (2001) Subcytotoxic attack by staphylococcal alpha-toxin activates NF-kappaB and induces interleukin-8 production. *Infect Immun* **69**:2630-2635.

Efstratiou A (1997) Pyogenic streptococci of Lancefield groups C and G as pathogens in man. *J Appl Microbiol* **83**:72S-79S.

Efstratiou A and Lamagni T (2016) Epidemiology of *Streptococcus pyogenes*, in *Streptococcus pyogenes : Basic Biology to Clinical Manifestations* (Ferretti JJ, Stevens DL and Fischetti VA eds), Oklahoma City (OK).

Freudenberg MA, Tchaptchet S, Keck S, Fejer G, Huber M, Schutze N, Beutler B and Galanos C (2008) Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. *Immunobiology* **213**:193-203.

Hancz D, Westerlund E, Valfridsson C, Aemero GM, Bastiat-Sempe B, Orning P, Lien E, Wessels MR and Persson JJ (2019) Streptolysin O Induces the Ubiquitination and Degradation of Pro-IL-1 $\beta$ . *J Innate Immun* **11**:457-468.

Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W and King GL (1992) Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A* **89**:11059-11063.

Kano F, Nakatsu D, Noguchi Y, Yamamoto A and Murata M (2012) A resealed-cell system for analyzing pathogenic intracellular events: perturbation of endocytic pathways under diabetic conditions. *PLoS One* **7**:e44127.

Kaplan EL, Rothermel CD and Johnson DR (1998) Antistreptolysin O and anti-deoxyribonuclease B titers: normal values for children ages 2 to 12 in the United States. *Pediatrics* **101**:86-88.

Kotby AA, Habeeb NM and Ezz El Elarab S (2012) Antistreptolysin O titer in health and disease: levels and significance. *Pediatr Rep* **4**:e8.

Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K and King GL (1997) Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *The Journal of clinical investigation* **100**:115-126.

Li J, Lin S, Vanhoutte PM, Woo CW and Xu A (2016) Akkermansia Muciniphila Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in Apoe<sup>-/-</sup> Mice. *Circulation* **133**:2434-2446.

Li J, Zhao F, Wang Y, Chen J, Tao J, Tian G, Wu S, Liu W, Cui Q, Geng B, Zhang W, Weldon R, Auguste K, Yang L, Liu X, Chen L, Yang X, Zhu B and Cai J (2017) Gut



microbiota dysbiosis contributes to the development of hypertension. *Microbiome* **5**:14.

Logsdon LK, Hakansson AP, Cortes G and Wessels MR (2011) Streptolysin O inhibits clathrin-dependent internalization of group A Streptococcus. *mBio* **2**:e00332-00310.

Lu SL, Kuo CF, Chen HW, Yang YS, Liu CC, Anderson R, Wu JJ and Lin YS (2015) Insufficient Acidification of Autophagosomes Facilitates Group A Streptococcus Survival and Growth in Endothelial Cells. *mBio* **6**:e01435-01415.

Magassa N, Chandrasekaran S and Caparon MG (2010) Streptococcus pyogenes cytolysin-mediated translocation does not require pore formation by streptolysin O. *EMBO Rep* **11**:400-405.

Mehta NN, Sheetz M, Price K, Comiskey L, Amrutia S, Iqbal N, Mohler ER and Reilly MP (2009) Selective PKC beta inhibition with ruboxistaurin and endothelial function in type-2 diabetes mellitus. *Cardiovasc Drugs Ther* **23**:17-24.

Meier M, Park JK, Overheu D, Kirsch T, Lindschau C, Gueler F, Leitges M, Menne J and Haller H (2007) Deletion of protein kinase C-beta isoform in vivo reduces renal hypertrophy but not albuminuria in the streptozotocin-induced diabetic mouse model. *Diabetes* **56**:346-354.

Mukohda M, Fang S, Wu J, Agbor LN, Nair AR, Ibeawuchi SC, Hu C, Liu X, Lu KT, Guo DF, Davis DR, Keen HL, Quelle FW and Sigmund CD (2019) RhoBTB1 protects against hypertension and arterial stiffness by restraining phosphodiesterase 5 activity. *The Journal of clinical investigation* **129**:2318-2332.

Mukohda M, Mizuno R and Ozaki H (2020) Increased Blood Pressure Causes Lymphatic Endothelial Dysfunction via Oxidative Stress in Spontaneously Hypertensive Rats. *Hypertension* **76**:598-606.

Niebauer J, Volk HD, Kemp M, Dominguez M, Schumann RR, Rauchhaus M, Poole-Wilson PA, Coats AJ and Anker SD (1999) Endotoxin and immune activation in chronic heart failure: a prospective cohort study. *Lancet* **353**:1838-1842.

Ohshiro Y, Ma RC, Yasuda Y, Hiraoka-Yamamoto J, Clermont AC, Isshiki K, Yagi K, Arikawa E, Kern TS and King GL (2006) Reduction of diabetes-induced oxidative stress, fibrotic cytokine expression, and renal dysfunction in protein kinase Cbeta-null mice. *Diabetes* **55**:3112-3120.

Santisteban MM, Qi Y, Zubcevic J, Kim S, Yang T, Shenoy V, Cole-Jeffrey CT, Lobaton GO, Stewart DC, Rubiano A, Simmons CS, Garcia-Pereira F, Johnson RD, Pepine CJ and Raizada MK (2017) Hypertension-Linked Pathophysiological Alterations in the Gut. *Circulation research* **120**:312-323.

Stassen M, Muller C, Richter C, Neudorfl C, Hultner L, Bhakdi S, Walev I and Schmitt E (2003) The streptococcal exotoxin streptolysin O activates mast cells to produce tumor necrosis factor alpha by p38 mitogen-activated protein kinase- and protein kinase C-dependent pathways. *Infect Immun* **71**:6171-6177.

Tabit CE, Shenouda SM, Holbrook M, Fetterman JL, Kiani S, Frame AA, Kluge MA, Held A, Dohadwala MM, Gokce N, Farb MG, Rosenzweig J, Ruderman N, Vita JA and Hamburg NM (2013) Protein kinase C-beta contributes to impaired endothelial insulin signaling in humans with diabetes mellitus. *Circulation* **127**:86-95.

Toral M, Robles-Vera I, de la Visitacion N, Romero M, Yang T, Sanchez M, Gomez-Guzman M, Jimenez R, Raizada MK and Duarte J (2019) Critical Role of the Interaction Gut Microbiota - Sympathetic Nervous System in the Regulation of Blood Pressure. *Front Physiol* **10**:231.

Uchiyama S, Dohrmann S, Timmer AM, Dixit N, Ghochani M, Bhandari T, Timmer JC, Sprague K, Bubeck-Wardenburg J, Simon SI and Nizet V (2015) Streptolysin O Rapidly Impairs Neutrophil Oxidative Burst and Antibacterial Responses to Group A Streptococcus. *Front Immunol* **6**:581.

Vita GM, De Simone G, Leboffe L, Montagnani F, Mariotti D, Di Bella S, Luzzati R, Gori A, Ascenzi P and di Masi A (2020) Human Serum Albumin Binds Streptolysin O (SLO) Toxin Produced by Group A Streptococcus and Inhibits Its Cytotoxic and Hemolytic Effects. *Front Immunol* **11**:507092.

Wiedermann CJ, Kiechl S, Dunzendorfer S, Schratzberger P, Egger G, Oberhollenzer F and Willeit J (1999) Association of endotoxemia with carotid atherosclerosis and cardiovascular disease: prospective results from the Bruneck Study. *J Am Coll Cardiol* **34**:1975-1981.

Yang T, Santisteban MM, Rodriguez V, Li E, Ahmari N, Carvajal JM, Zadeh M, Gong M, Qi Y, Zubcevic J, Sahay B, Pepine CJ, Raizada MK and Mohamadzadeh M (2015) Gut dysbiosis is linked to hypertension. *Hypertension* **65**:1331-1340.

### *Footnotes*

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### *CONFLICT OF INTEREST DISCLOSURE*

The authors have declared that no conflict of interest exists.

**Figure 1. Change in vascular contractile activity in response to SLO.**

Isometric tension experiments were performed using thoracic aortic rings from Wistar rats treated *ex vivo* with or without SLO (1–1000 ng/mL) for 30 min. Cumulative concentration-response curves for phenylephrine (1 nM–30  $\mu$ M) (A, n=4, SLO 1-100 ng/ml) (D, n=4, SLO 300-1000 ng/ml), serotonin (5-HT, 10 nM–30  $\mu$ M) (B, n=4), or angiotensin II (Ang II, 0.1 nM–3  $\mu$ M) (C, n=4) were recorded. Data are presented as mean  $\pm$  SEM.

**Figure 2. Change in vasodilation in response to SLO.**

A-E) Isometric tension experiments were performed using thoracic aortic or 2nd-branch mesenteric arterial rings from Wistar rats treated *ex vivo* with or without SLO (1–1000 ng/mL) for 30 min. Cumulative concentration-response curves for acetylcholine (ACh, 0.1 nM–3  $\mu$ M) (A, C) or sodium nitroprusside (SNP, 0.1 nM–3  $\mu$ M) (B, D) in thoracic aorta (A, n=4-6) (B, n=4) or 2nd-branch mesenteric artery (C, n=4-5) (D, n=4) from Wistar rats. In presence of 1% human serum albumin, cumulative concentration-response curves for ACh (0.3 nM–30  $\mu$ M) was performed (E, n=5-6). F-G) Western blot detecting the indicated proteins in aorta treated with SLO (100 ng/mL) (representative of 8 experiments). Quantification of Western blots for p-eNOS Thr495 (Panel F) and total eNOS in blots such as the representative shown in Panel E (Panel G). p-eNOS refers to the phosphorylated form of eNOS. Data are presented as mean  $\pm$  SEM. \*P<0.05 vs. Control, N.S.; not significance.

**Figure 3. Role of p38 MAPK and PKC in SLO-induced endothelial dysfunction.**

A) Cumulative concentration-response curves for ACh (0.3 nM-3  $\mu$ M) in thoracic aorta from Wistar rat (n=5). Aortic rings were treated with SB203580 (10  $\mu$ M, 30 min) then SLO (100 ng/ml, 30 min) for 30 min. B-C) Western blot detecting the indicated proteins in aorta isolated from Wistar rat treated with SLO (100 ng/ml) (representative of 11 experiments). Quantification of Western blots for p-p38 (Panel A) and total p38 such as the representative shown (Panel B). p-p38 refers to the phosphorylated form of p38. D-F) Aortas were pretreated with vehicle (control) or the pan PKC inhibitor (Ro 31-8220, 100 nM) (D, n=6), the PKC $\beta$  inhibitor (LY333531, 300 nM) (E, n=5) or the selective PKC $\beta$ 2 inhibitor (CGP53353, 10  $\mu$ M) (F, n=5) for 30 min before exposure to SLO (100 ng/ml) or control treatments. Isometric tension experiments were then performed with ACh (0.3 nM-3  $\mu$ M). G-H) Western blot detecting the indicated proteins in SLO (100 ng/ml)-treated aorta pretreated with Ro31-8222 (100 nM, 30 min) or CGP53353 (10  $\mu$ M, 30 min) (representative of 9 experiments). Quantification of Western blots for p-eNOS Thr495 (Panel G) and total eNOS such as the representative shown. p-eNOS refers to the phosphorylated form of eNOS. I-J) Western blot detecting the indicated proteins in aorta treated with SLO (100 ng/ml) (representative of 10 experiments). Quantification of Western blots for Phospho-PKC substrates (Panel I) such as the representative shown. p-PKC substrates refer to the phosphorylated PKC substrates. Data are presented as mean  $\pm$  SEM. \*P<0.05 vs. Control, #P<0.05 vs. SLO.

**Figure 4. Change in cardiovascular function in response to SLO in Wistar rats.**

A-F) Mean blood pressure (MBP), heart rate (HR), and pulse pressure (PP) measured using arterial catheter in adult Wistar rats. A-C) SLO (0.64–640 ng/kg, 10–15 min) was injected via the femoral vein. D-F) Cumulative concentration-response

curves to MBP (D, n=5), HR (E, n=4), and PP (F, n=5) for ACh (0.02–20 µg/kg) were recorded after SLO (640 ng/kg, 10–15 min) was injected via the femoral vein. Data are presented as mean ± SEM. \*P<0.05 vs. Control.

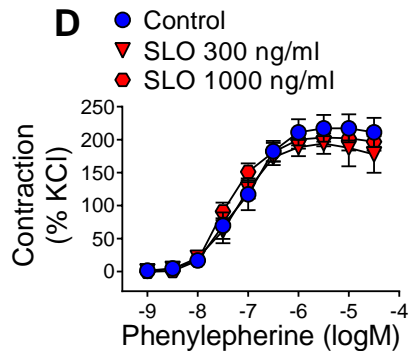
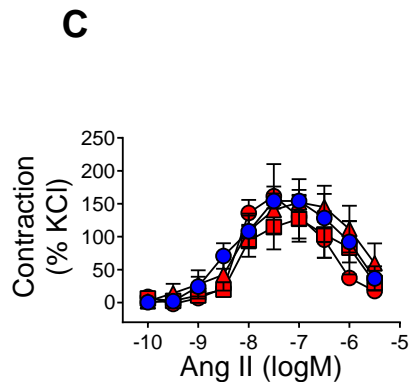
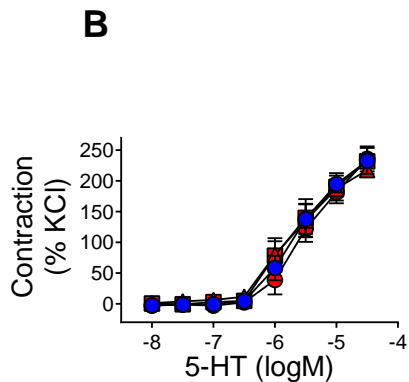
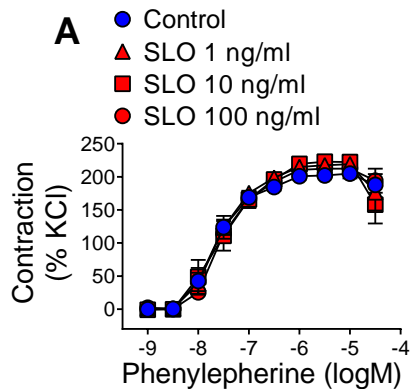
**Figure 5. Plasma levels of anti-SLO IgG in WKYs and SHR.**

A) SBP measured using tail-cuff plethysmography in 10- to 15-week-old Wistar Kyoto rats (WKYs) and spontaneous hypertensive rats (SHRs). B) Ratio of heart weight to body weight (HW/BW) in WKYs and SHRs. C) Plasma levels of anti-SLO IgG in WKYs and SHRs. Data are presented as mean ± SEM. \*P<0.05 vs. WKY.

**Figure 6. Change in cardiovascular function in response to SLO in SHRs.**

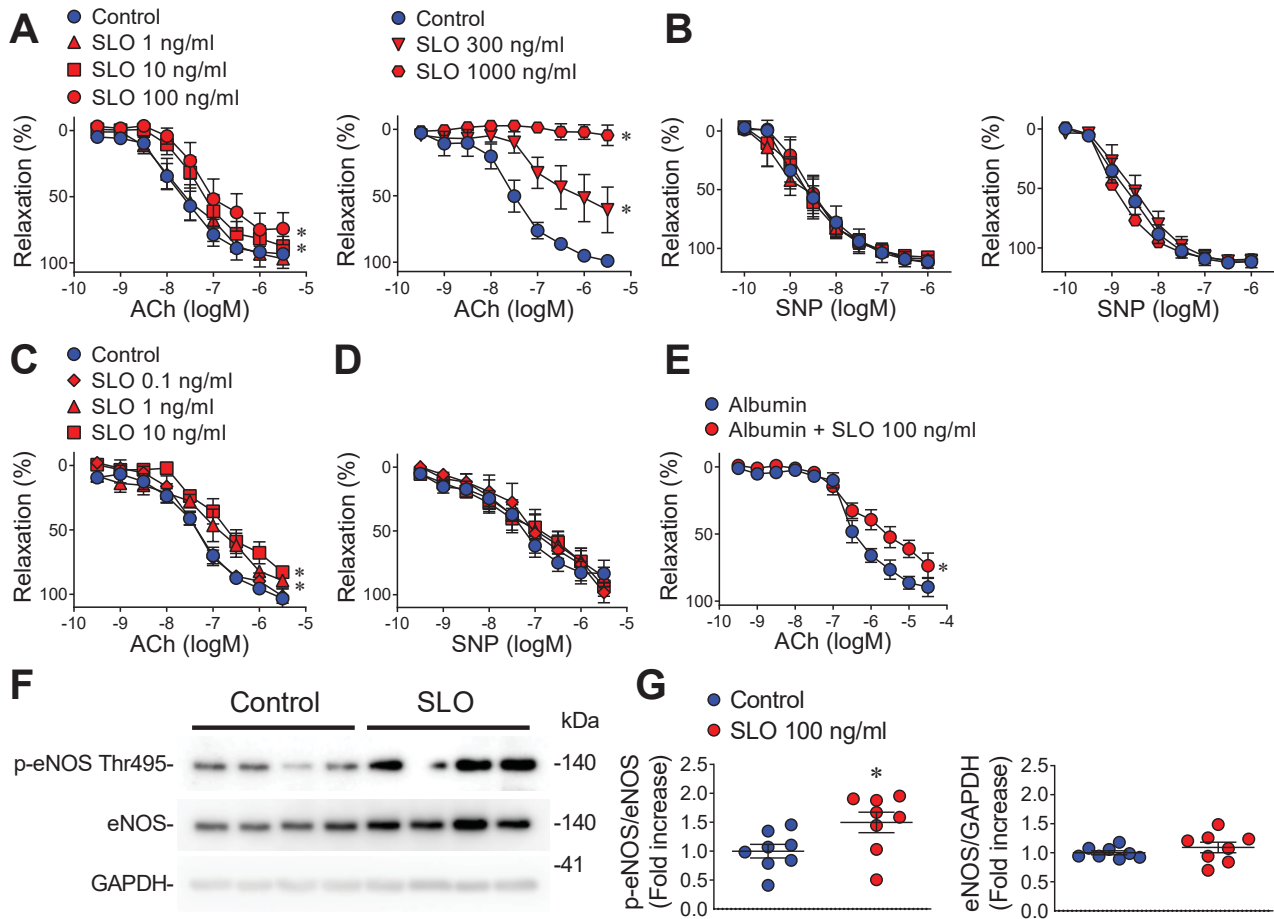
A-F) MBP, HR, and PP measured using arterial catheter in adult SHRs. A-C) SLO (0.64–640 ng/kg, 10–15 min) was injected via the femoral vein. D-F) Cumulative concentration-response curves to MBP (D, n=4), HR (E, n=4), and PP (F, n=4) for ACh (0.02–20 µg/kg) were recorded after SLO (640 ng/kg, 10–15 min) was injected via the femoral vein. Data are presented as mean ± SEM. \*P<0.05 vs. SHR+Control.

# Figure 1

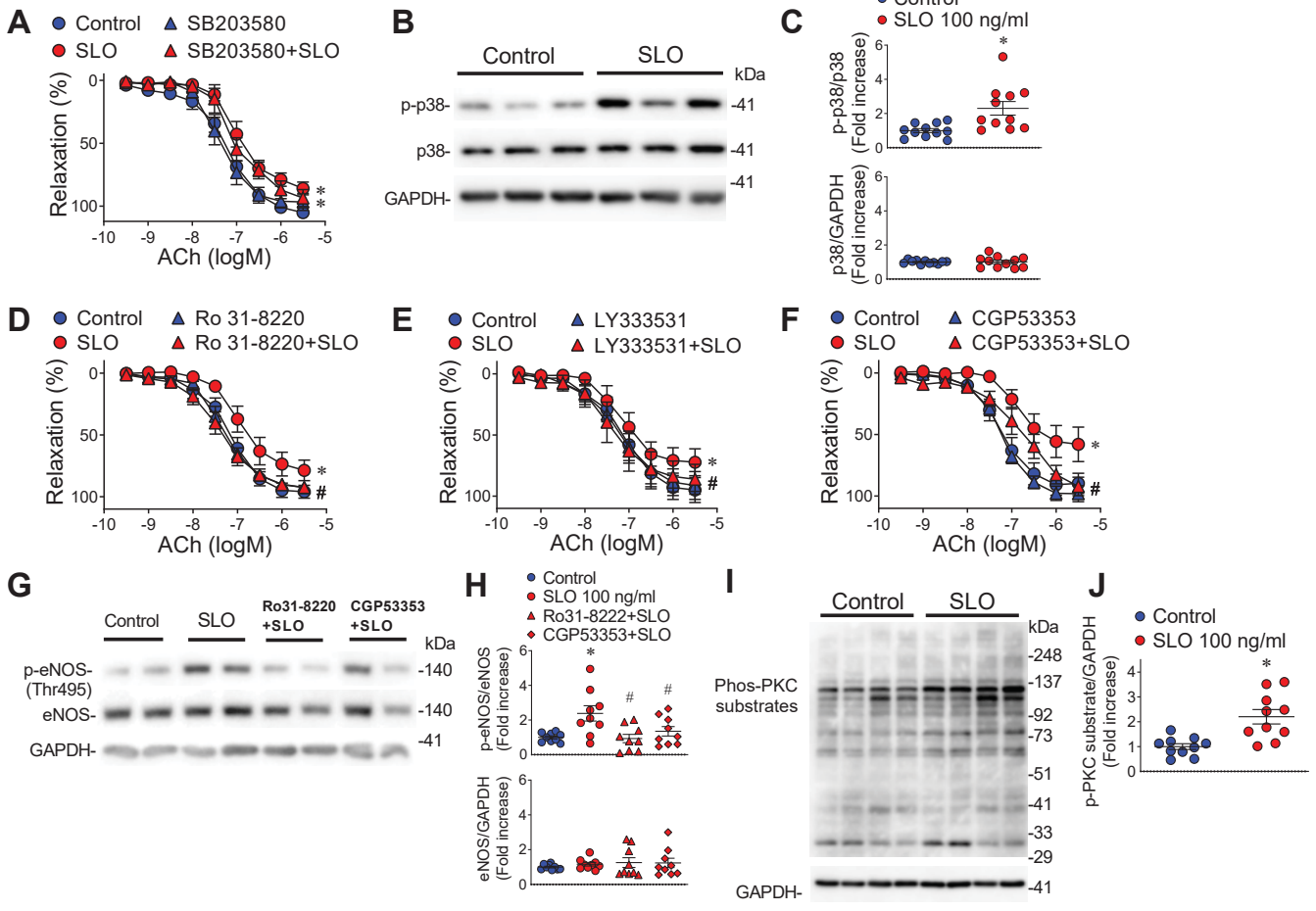




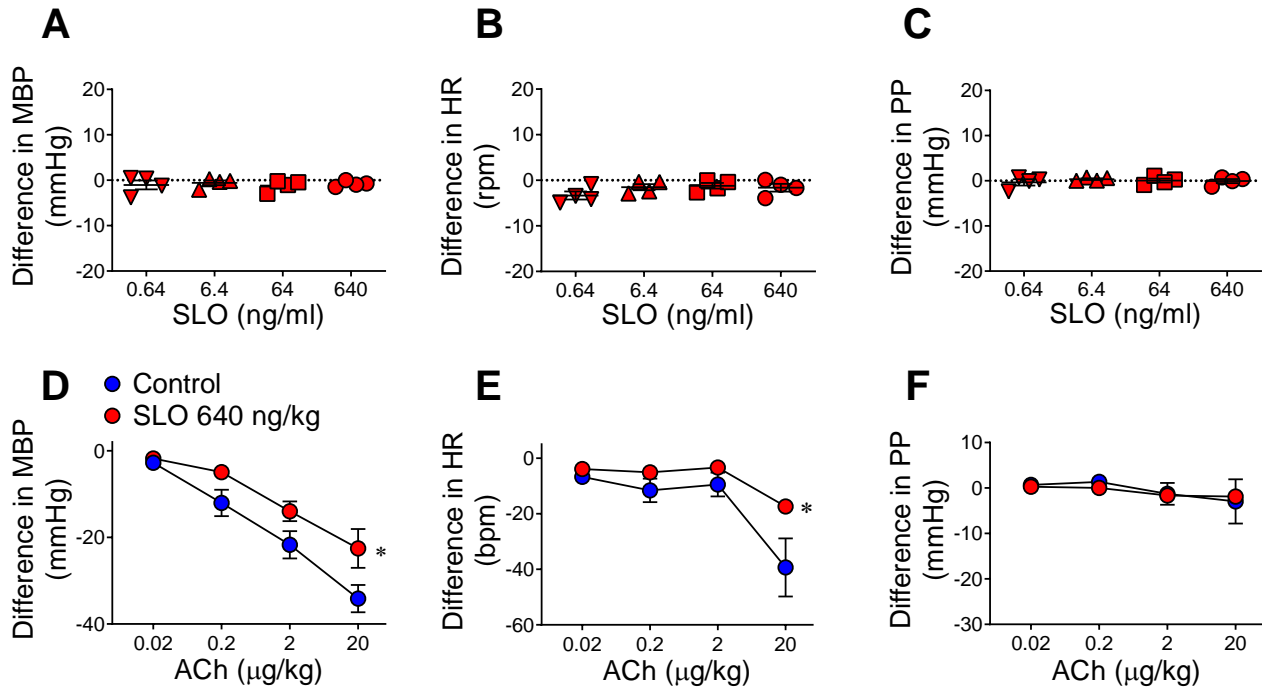
**Figure 2**



**Figure 3**

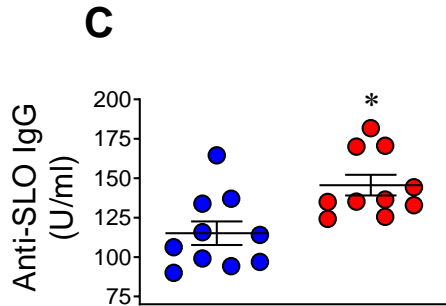
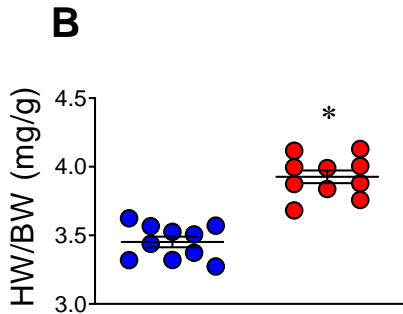
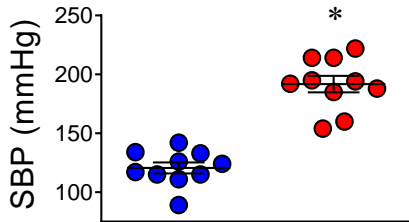


# Figure 4



# Figure 5

**A** ● WKY 10-15 wks  
● SHR 10-15 wks



**Figure 6**

