JPET/2003/57422

p38 MAPK Inhibitors Ameliorate Target Organ Damage in Hypertension: Part 1, p38 MAPK-Dependent Endothelial Dysfunction and Hypertension

Haisong Ju*, David J. Behm*, Sandyha Nerurkar, Marianne E. Eybye, Robin E. Haimbach, Alan R. Olzinski Stephen A. Douglas, Robert N Willette

Departments of Investigative and Cardiac Biology and Vascular Biology
Cardiovascular and Urogenital Center of Excellence in Drug Discovery
GlaxoSmithKline
709 Swedeland Road, King of Prussia, PA 19406, U.S.A.

Running Title: p38 MAPK-dependent hypertension

Corresponding author: Robert N Willette, PhD
Department of Investigative and Cardiac Biology, UW2510
GlaxoSmithKline
709 Swedeland Road, PO Box 1539
King of Prussia, PA 19406, USA
Tel.: (610) 270 6052
Fax.: (610) 270 5080
e-mail: robert_n_willette@gsk.com
*contributed equally to this study

# text pages	32
# of Tables	1
# of Figures	8
# of References	28
# words in Abstract	202
# words in Introduction	262
# words in Discussion	764

Abbreviations: ICAM-1, intercellular adhesion molecule-1; LPS, lipopolysaccharide; ND, normal chow diet; p38 mitogen activated protein kinase, p38 MAPK; SFD, salt/fat diet; SHRSP, spontaneously hypertensive stroke prone rats.

Section: Cardiovascular

Abstract

Numerous mediators, believed to play a role in endothelial dysfunction (e.g. neurohormones, cytokines, hypoxia and stretch), have been shown to activate p38 MAPK in a variety of cell types. The purpose of the present study was to examine the regulation of p38 MAPK in endothelium and its role in endothelial dysfunction and salt-sensitivity. In cultured human umbilical vein endothelial cells (HUVECs), TNF-α and LPS increased phosphorylation of p38 MAPK (P-p38 MAPK) and increased ICAM-1 expression. Preincubation with highly selective p38 MAPK inhibitors, SB-239063AN or SB-239063, dose-dependently reduced ICAM-1 expression in HUVECs. In stroke-prone spontaneously hypertensive rats (SHRSP), P-p38 MAPK was localized by immunohistochemistry to the aortic endothelium and adventitia but was undetectable in aortae from normotensive rats. Introduction of a salt-fat diet (SFD) to the SHRSP strain induced endothelial dysfunction (*ex vivo* vascular reactivity analysis), microalbuminuria and an increase in blood pressure within 4 weeks. Chronic dietary dosing (approx. 100mg/kg/day) with SB-239063AN inhibited the SFD diet-induced hypertension. In addition, delayed treatment also significantly improved survival and restored NO-mediated endothelium–dependent relaxation in SFD-SHRSPs with established endothelial dysfunction. These results suggest an important role for p38 MAPK in endothelial inflammation and dysfunction as well as providing the first evidence for p38 MAPK-dependent hypertension.

Endothelial-derived factors normally play an important role in maintaining vascular homeostasis by regulating vasomotion, inflammation, thrombosis, and smooth muscle proliferation (Kinlay et al., 2001; Schiffrin, 2002). In contrast, endothelial dysfunction has been implicated in the pathogenesis and clinical course of major cardiovascular disease (Verma and Anderson, 2002; Schiffrin, 2002). In particular, endothelial dysfunction is associated with atherosclerosis and hypertension, as well as diabetes and dyslipidemia. (Ross, 1999; Shimokawa, 1999).

The mechanisms underlying endothelial dysfunction are not well understood but evidence suggests that growth factors, angiotensin II, oxidative stress, elevated LDL, and cytokines are contributing factors. All have been shown to activate p38 MAPK signaling in a variety of cell types with only limited supporting evidence in endothelial cells and endothelial dysfunction (Raitakari and Celermajer, 2000) (Zhu et al., 2001) (Huot et al., 1997) (Read et al., 1997) (Touyz et al., 2001) (Pietersma et al., 1997) (Tamura et al., 1998).

The first part of this two-part study examined the hypothesis that p38 MAPK plays an important role in endothelial dysfunction associated with salt-sensitive hypertension and that treatment with a selective p38 MAPK inhibitor would restore endothelial-dependent vasorelaxation. Specifically, the regulation of p38 MAPK and the downstream expression of adhesion molecules were examined in human umbilical vein endothelial cells (HUVECs). In addition, the effects of treatment with selective p38 MAPK inhibitors (SB-239063 and SB-239063AN) on endothelial-dependent vasorelaxation, blood pressure, microalbuminuria and survival were determined in the

spontaneously hypertensive stroke prone rat (SHRSP). A separate second part of this study examined the role of p38 MAPK in renal dysfunction associated with hypertensive salt-sensitivity (current issue).

Methods

Endothelial cell culture and treatment:

Human umbilical vein endothelial cells (HUVECs) were purchased from BioWhittaker (Walkersville, MD) and were cultured in EGM-2 basal medium supplied with 2% FBS and growth factors (as recommended by BioWhittaker) in a humidified incubator at 37°C with 5% CO_2. HUVECs were grown in 100 mm culture dishes, passaged using 0.05% trypsin/1 mM EDTA solution and experiments were performed in confluent HUVECs, passage 5-6, maintained in EGM-2 basal medium supplied with 0.5% FBS without growth factors. Temporal p38 MAPK activation studies were performed in HUVECs stimulated with TNF-α at a final concentration of 1 ng/ml for 5 to 60 min. When examining expression of adhesion molecules, HUVECs were pretreated with various concentrations of a selective p38 MAPK inhibitor (Fig. 1), SB-239063AN (1-(1,3-dihydroxyprop-2-yl)-4-(4-fluorophenyl)-5-[2-phenoxypyrimidin-4-yl] imidazole), for 1h before stimulation with TNF-α (1 ng/ml) or 100 ng/ml LPS 011:B4 (Sigma, St. Louis, MO) for 6 hr (Kim et al., 2001). HUVECs were then washed in cold PBS and disrupted using lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 μg/ml leupeptin (Cell Signaling Technology Inc., Beverly, MA). Cell lysates were used for Western blot analysis. All HUVEC experiments were repeated at least three times and representative blots are presented.

Western blotting:

Harvested HUVECs were sonicated twice for 5s and were centrifuged at 14000 X g for 10 min. The resulting supernatant was used for protein determination (DC protein assay, Bio-Rad, Hercules, CA) and subsequent Western blot analysis. Briefly, 30μg protein was resolved on 8-16% precasted SDS-PAGE tris-glycine gel (Invitrogen, Carlsbad, CA) and protein was transferred to a PVDF membrane. Non-specific binding was blocked by incubation of the membrane with blocking solution (Zymed, South San Francisco, CA) at room temperature for 1h. The membrane was then incubated with primary antibodies in blocking solution overnight at 4°C followed by incubating with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1h. Primary antibodies used in the current study were P-p38 MAPK (Cell Signaling Technology Inc., Beverly, MA), p38 MAPK (Cell Signaling Technology Inc.), ICAM-1 (Santa Cruz Biotechnology, Stan Cruz, CA), and PECAM-1 (Santa Cruz Biotechnology) in 500-1:1000 dilution. Immunoreactive bands were detected using chemiluminescence detection reagent (Amersham International, Arlington Heights, IL).

SHRSP studies:

Spontaneously hypertensive stroke prone rats (SHRSP), obtained from the National Institutes of Health (NIH; Bethesda), were bred in the Department of Laboratory Animal Science at GlaxoSmithKline (King of Prussia). Age-matched normotensive rats (Wistar-Kyoto (WKY) and Sprague-Dawley (SD)) were purchased from Charles River Laboratories (Wilmington, MA). Experiments were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (NIH Publication 85-

23) and experimental protocols were reviewed and approved by the GSK Animal Care and Use Committee.

Endothelial dysfunction and microalbuminuria time-course study:

Vascular reactivity was examined in SHRSPs on a normal chow diet (n=18) and in normotensive WKY rats (n=18) in subsets (n=6) at 0 weeks (10-12 weeks of age), 4 and 8 weeks. SHRSPs on a salt/fat diet (SFD) were examined at 4 and 8 weeks after starting the diet (n=5-6 per group). The SFD has been described previously and contains 24.5% fat in the food and 1% NaCl in the drinking water provided *ad libitum* (Barone et al., 1996). A 24 hr urine collection was obtained weekly from each SHRSP animal and albumin excretion (mg/day) was determined using an immunoturbidometric assay optimized for the determination of rat urinary albumin in an autoanalyzer format (Kamiya Biomedical, Seattle, WA., KRA-010/020).

Treatment study:

A colony of 62 male SHRSPs, aged 9-12 weeks, was divided and assigned to one of three groups. All animals were placed on the normal chow diet (NIH-07 diet) while baseline/entry monitoring was completed and then assigned to diet or treatment groups. One SHRSP group was maintained on the normal chow diet (ND) throughout the study (n=10). The other two groups received SFD for a "run-in" period until 10% mortality (about 6 weeks). Then, 18 SHRSPs in the SFD group received diet containing 1500 ppm of SB-239063AN (see Fig 1 for chemical structure), a highly selective p38α/β MAPK inhibitor, for the duration of the study (12 weeks). The remaining

SHRSPs in the SFD group continued to receive the SFD until the end of the study (n=28). Mean age and body weight was consistent in all groups. SFD-SHRSPs have an accelerated mortality rate and were promptly euthanized when signs of morbidity were noted. These signs may include piloerection, lack of grooming, hypersensitivity to sound or touch, loss of appetite in the setting of cachexia, ataxia, decreased movement and convulsive movements (Behr, et al., 2001).

Assessment of vascular reactivity:

All animals were randomly selected from the time-course or treatment groups (n=5-6 per group) and anesthetized with 5% isoflurane in O_2 and their proximal descending thoracic aortae were isolated and prepared for vascular reactivity studies as described previously (Behm et al, 2002). Vascular ring segments (2-3 mm) were suspended in 10mL tissue baths containing Krebs of the following composition (mM): NaCl, 112.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; dextrose, 11.0. The Krebs solution was maintained at 37 ± 1^oC and aerated with $95\%O_2$:$5\%CO_2$ (pH 7.4). Changes in isometric force were measured under a resting tension (1g) using FT03 force-displacement transducers (Grass Instruments, Quincy, MA) coupled to Model 7D polygraphs. Following a 60 min equilibration period, the vessels were treated with standard concentrations of KCl (60 mM) and norepinephrine (1 μM). Vascular contractility was evaluated by examining the cumulative concentration-contraction relationship elicited by adding half-log increments (0.1 nM-10 μM) of norepinephrine to the tissue baths. Endothelium-dependent vasorelaxation was assessed in vessels precontracted with an approximate EC_{80} norepinephrine (100 nM) by comparing

concentration-related carbachol-induced relaxation. Cumulative concentration-response curves to carbachol were obtained by adding half-log increments (1 nM - 100 μM) of the relaxant to the tissue. In a similar manner, endothelium-independent vasorelaxation was demonstrated using sodium nitroprusside (SNP) at 0.1 nM - 10 μM. Norepinephrine contraction was expressed as a percentage of the contractile response to 60 mM KCl and carbachol and SNP relaxation was expressed as percent reversal of the norepinephrine precontraction response.

Blood pressure telemetry studies:

Male SHRSP rats (5-6 weeks of age) maintained on a normal powdered diet (Purina Diet 5001) were anesthetized with 2% isoflurane anesthesia and a telemetry transmitter (TA11PA-C40, Data Sciences International, St.Paul, MN) was implanted. The transmitter catheter was inserted into the femoral artery and advanced threaded to the lower abdominal aorta. At approximately 8 weeks of age, animals were fed one of three diets: A) salt/fat diet (SFD) composed of a high fat diet (24.5% fat, Zeigler Brothers, Gardner, PA) plus 1% NaCl drinking water (n=10 rats); B). high fat diet containing SB-239063 (trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl methoxypyridimidin-4-yl)imidazole) (Fig 1) at 1200 ppm plus 1% NaCl drinking water (n=6 rats); C) normal powdered diet plus tap water (n=5 rats). Baseline measurements of systolic and diastolic blood pressure, heart rate and activity were obtained one week prior to starting special diets. Recordings were obtained each week thereafter for a continuous period of 48 hours with data acquisition of 10-second averages every 5

minutes. Body weight and fluid consumption were measured daily. All animals were monitored for 6 weeks.

Pilot studies were performed in SFD-SHRSP to establish dosing regimens sufficient to attain plasma peak and trough concentrations of approximately 1.5 and 0.5 uM, respectively, for SB-239063AN and SB-239063. In the present chronic studies, plasma samples were prepared for drug analysis following 3 weeks of treatment (sampling time was10-11 AM).

Phospho-p38 MAPK immunohistochemistry:

Thoracic aortae from age-matched (26-28 weeks) SHRSP and SD rats (n=5 per group) were rapidly harvested and fixed in 10% formalin overnight and then kept in 70% ethanol until paraffin embedding, sectioning and staining for phospho-p38 MAPK. Slides were deparaffinized, rehydrated and placed in phosphate buffered saline (PBS) with 0.1% Tween-20. Sections were stained using streptavidin-horseradish peroxidase labelling system on the DAKO Autostainer (Carpenteria, CA). Briefly, the slides were exposed to 3% hydrogen peroxide for 15 min. and non-specific binding was blocked with normal serum at 1:50. Sections were incubated with the primary antibodies for 45 min followed by a 30 min incubation with biotinylated secondary antibodies. Slides were incubated in 1:200 dilution of streptavidin-horseradish peroxidase (DAKO) for 15 min followed by incubation with substrate 3',3'-diaminobenzidine for 5 min. The slides were counterstained with hematoxylin, dehydrated and coverslipped. A monoclonal antibody (1:10

dilution) was used for the localization of phospho-p38 MAPK (Sigma, St. Louis, MO).

Drugs:

Potency, selectivity, *in vivo* and *in vitro* efficacy profiles and pharmacokinetic properties were similar for SB-239063 and SB-239063AN (Fig. 1). Both compounds have been cross-screened for activity in a panel of 56 protein kinases and IC_{50}s have been generated where possible. SB-239063 and SB-239063AN are potent p38 inhibitors (IC_{50} = 46 nM and 36 nM, respectively) with selectivity of 25-fold and 4-fold vs. JNK2, respectively, and $\geq$600-fold vs. all other kinases tested. In an LPS-stimulated human whole blood assay, SB 239063 and SB-239063AN inhibited TNF-α production with IC_{50}s of 1.0 and 0.3 uM, respectively.

Statistical analysis:

All summary values are expressed as the mean $\pm$ standard error of the mean. Chi-square analysis was performed for quantal responses at each time interval. Multiple comparisons of the means were made by ANOVA followed by *post hoc* analysis with the Bonferroni correction for multiple comparisons. All statistical analyses were done using InStat (GraphPad Software, San Diego, CA) and $p \leq 0.05$ was considered to be significant.

Results

Activation of endothelial p38 MAPK in vitro and in vivo:

The activation of p38 MAPK was examined by Western blot analysis of phospho-p38 (P-p38) MAPK in cultured HUVECs. As shown in Fig. 2A, incubation of HUVECs with a proinflammatory cytokine, TNF-α (1 ng/ml), produced a rapid increase in p38 MAPK phosphorylation which persisted for approximately 30 mins. LPS induced a similar temporal activation pattern in HUVECs (data not shown). In contrast, total p38 MAPK was not altered by TNF-α treatment.

To investigate endothelial p38 MAPK activation *in situ,* P-p38 MAPK immunohistochemistry was performed on thoracic aortae obtained from age-matched (16-18 weeks) SHRSPs and normotensive Sprague-Dawley rats. Striking differences in phospho-p38 MAPK immunoreactivity were noted in the hypertensive and normotensive strains. P-p38 MAPK immunoreactivity was localized primarily to the endothelium and adventitial/medial boarder in the SHRSP aortae (Fig. 2C). In contrast, little or no phospho-p38 MAPK was evident in aortae from normotensive SD and WKY rats (Fig. 2B)

Effect of p38 MAPK inhibition on adhesion molecules expression in HUVECs:

The effects of a selective p38 MAPK inhibitor, SB-239063AN, on adhesion molecule expression induced by inflammatory mediators were examined in cultured HUVECs. Both LPS and TNF-α induced a dramatic increase in ICAM-1 expression after a 6-hour incubation (Fig. 3A&B). Preincubaton with SB-239063AN (1-10 μM) produced a dose-dependent inhibition of HUVEC ICAM-1 expression induced by LPS or

TNF-α stimulation (Fig 3A&B). In contrast, neither LPS nor TNF-α altered PCAM expression in HUVECs.

Endothelial dysfunction and albuminuria in the SFD-SHRSP:

In order to assess the time-course of endothelial dysfunction in SFD-SHRSPs, *ex vivo* vascular reactivity of the thoracic aorta was compared at 0, 4 and 8 weeks of study in WKY (normal diet) and SHRSPs (normal or salt/fat diet). On study week 0, there was no significant difference in vascular reactivity in age-matched WKY or SHRSP rats, i.e. the potency and efficacy of alpha-adrenoceptor mediated contraction (phenylephrine), endothelial-dependent relaxation (carbachol) and endothelial-independent relaxation (SNP) were equivalent (Fig. 4). However, the introduction of the SFD to SHRSPs resulted in a significant reduction in the efficacy of endothelial-dependent relaxation at 4 and 8 weeks as compared to age-matched WKYs (Fig 4). Little or no effect on endothelial-dependent relaxation was observed in the ND-SHRSP or ND-WKY groups (Fig. 4). No significant changes in the phenylephrine and SNP efficacy (Rmax) were noted in any of the groups (Table 1). These results indicate that the introduction of a SFD induces significant endothelial dysfunction within 4 weeks.

The introduction of a SFD was also associated with a significant increase in albuminuria in SHRSPs. Time-dependent increases in albuminuria were observed following 4 weeks on the SFD (Fig. 5). Only slight increases in albuminuria were observed in SHRSPs on a normal diet and no changes were noted in WKY (results not shown). The results indicate that endothelial dysfunction precedes the onset of detectable microalbuminuria in the SFD-SHRSP and suggests a causal relationship. A

more detailed description of changes in renal function can be found in the second part of this series in the current issue.

Restoration of endothelial function and survival benefit by chronic/delayed treatment with p38 MAPK inhibitor:

A study was performed in the SFD-SHRSP model of endothelial dysfunction to determine if chronic treatment with a p38 MAPK inhibitor could restore endothelial function. SHRSP were placed on a SFD and randomized in 2:1 fashion to no treatment or treatment with SB-239063AN (1500 ppm in the diet) when 10% mortality was observed (approximately 6 weeks after starting the SFD). This model exhibits severe endothelial dysfunction and target organ damage during this time period. The introduction of treatment with SB-239063AN induced a surprisingly rapid improvement in survival (Fig. 6). In addition, 12 weeks of treatment restored endothelial-dependent relaxation to levels comparable to SHRSPs that had been maintained on a ND throughout the study (Fig 7, Table 2). Plasma concentrations of SB-239063AN after 3 weeks of treatment were 386 $\pm$ 25 ng/ml, well within the peak/trough range defined in pilot exposure studies.

In vascular reactivity studies performed as above, SB-239063 and SB-239063AN had no significant effects on contraction or relaxation parameters when added directly to the tissue bath in concentration up to 10 uM (data not shown).

SFD-induced hypertension is attenuated by treatment with a selective p38 MAPK inhibitor:

Chronic blood pressure telemetry studies were performed to examine the effects of treatment with a p38 MAPK inhibitor on SFD-induced progessive hypertension in SHRSPs. Introduction of the SFD increased mean arterial blood pressure (MAP) 45-50 mmHg within 4 weeks. SB-239063 (1200 ppm) added to the diet significantly reduced SFD-induced hypertension and MAP in the treatment group was similar to SHRSPs maintained on a ND (Fig. 8). The study was terminated at 5 weeks because of mortality in the untreated group. Plasma concentrations of SB-239063 after 3 weeks of treatment were 419 $\pm$ 56 ng/ml, well within the peak/trough range defined in pilot exposure studies. Similar chronic treatment with SB-239063 had no significant effect on MAP in the salt-insensitive SHR strain (data not shown).

Discussion

The salt-sensitive phenotype in the SHRSP relates to an enhanced BP response to salt and acceleration/potentiation of hypertension-induced target organ damage (Barone et al., 2001;Liu et al., 1999;Ma et al., 2001;Griffin et al., 2001). In the present study, the regulation of endothelial p38 MAPK and its role in BP regulation and endothelial dysfunction were examined in this model and in cultured HUVECs. Proinflammatory cytokines enhanced phosphorylation of endothelial p38 MAPK in HUVECs and phosphorylated p38 MAPK was localized to the endothelium, outer media and adventitia in blood vessels from SHRSPs. Treatment with selective inhibitors of p38 MAPK attenuated the expression of ICAM adhesion molecules in HUVECs. In SHRSPs with established target organ damage (endothelial and renal dysfunction) induced with a SFD, chronic treatment with p38 MAPK inhibitors restored endothelial-dependent vasorelaxation and attenuated the morbidity/mortality. In addition, chronic treatment with a p38 MAPK inhibitor abolished the gradual increase in BP induced by a SFD. These results suggest, for the first time, a role for p38 MAPK in the pathogenesis of hypertension.

The SHRSP strain exhibits differential salt-sensitivity (Griffin et al., 2001) and, when placed on a salt/fat diet, represents is an aggressive hypertension model of accelerated target organ damage with characteristic pathology evident in the blood vessels, brain, heart, kidney, and retina (Barone et al., 2001). The introduction of the SFD induces a progressive increase in blood pressure in the SHRSP within 2 weeks with concomitant depression of endothelial-dependent vasorelaxation which is followed by evidence of renal dysfunction (microalbuminuria) and subsequent neurological

deficits (Behr et al., 2001; Ma et al., 2001). Thus, it appears that endothelial dysfunction (loss of bioavailable endothelial NO) contributes to the progression and maintenance of chronic hypertension and is a likely determinant of the prothrombotic state and target organ damage observed in this model (Behr et al., 2001; Ma et al., 2001; Yamashita et al., 2002).

Endothelial dysfunction in the SHRSP is associated with an accumulation of nitrotyrosine in the blood vessel wall suggesting rapid inactivation of endothelial NO and peroxynitrite production by excessive generation of reactive oxygen species (ROS). The major source of vascular ROS in hypertension is via NAD(P)H oxidase activation by mechanical factors, vasoactive agents, cytokines and growth factors (Touyz, 2003;Wilcox, 2002;Landmesser and Harrison, 2001). All of these stimuli activate p38 MAPK (kinase-dependent and redox-dependent signaling) which contributes to further generation of ROS via proinflammatory cytokine generation. The present results suggest that inappropriate activation of the p38 MAPK pathway contributes to an excess ROS and subsequent reduction in NO bioavailability. Furthermore, the process is reversible by treatment with a selective p38 MAPK inhibitor.

In addition to the proposed role in NO scavenging, evidence suggests that redox-dependent and kinase-dependent signaling through p38 MAPK plays an important role in vascular inflammation and remodeling processes. In this regard, p38 MAPK activation was observed in medial and adventitial lamina of remodeling blood vessels from hypertensive animals. These results are not limited to hypertension and appear to be consistent with sustained activation of p38 MAPK noted in remodeling blood vessels following mechanical injury and hypercholesterolemia (Ju et al., 2002).

Chronic treatment with selective p38 MAPK inhibitors limit vascular remodeling in these normotensive vascular injury models suggesting that p38 MAPK plays a broad role in vasculopathies linking cardiovascular disorders such as hypertension and atherosclerosis.

The mechanism(s) of salt-sensitivity in the SHRSP are unknown, however, brief exposure to a high salt diet has been shown to induce endothelial dysfunction independent of changes in blood pressure (Liu et al., 1999). In addition, high salt has been shown to increase ROS and the activity of ROS generating enzymes NAD(P)H oxidase and xanthine oxidase (Lenda and Boegehold, 2002a; Lenda and Boegehold, 2002b). These observations suggest that the initiating events in salt-sensitivity serve to reduce bioavailable NO (Hamilton et al., 2001). The role of p38 MAPK in these initiating events has not been evaluated directly, but is implicated in the present study by the attenuation of salt-induced hypertension induced by treatment with selective p38 MAPK inhibitors. The present study also suggests a relationship between endothelial dysfunction and end-organ damage such as renal dysfunction and stroke. However, it is unclear whether endothelial protection is the sole mechanism by which p38 MAPK inhibitors ameliorate hypertensive end organ damage.

In conclusion, salt sensitivity in the SHRSP is associated with a progressive increase in blood pressure and concomitant endothelial and renal dysfunction, as well as enhanced morbidity and mortality. Treatment with a p38 MAPK inhibitor restored endothelial-dependent relaxation and reduced the mortality rate. Salt-sensitive hypertension was also ameliorated by treatment with a p38 MAPK inhibitor providing the first evidence for p38 MAPK-dependent hypertension.

Reference List

Barone FC, Nelson AH, Ohlstein EH, Willette RN, Sealey JE, Laragh JH, Campbell WG Jr, Feuerstein GZ. (1996) Chronic carvedilol reduces mortality and renal damage in hypertensive stroke-prone rats. *J Pharm Exp Ther.* **279**:948-955.

Barone FC, Coatney RW, Chandra S, Sarkar SK, Nelson AH, Contino LC, Brooks DP, Campbell WG, Jr., Ohlstein EH, and Willette RN (2001) Eprosartan reduces cardiac hypertrophy, protects heart and kidney, and prevents early mortality in severely hypertensive stroke-prone rats. *Cardiovasc.Res.* **50**:525-537.

Behm DJ, Herold CL, Ohlstein EH, Knight SD, Dhanak D, Douglas SA (2002) Pharmacological characterization of SB-710411 (Cpa-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Cpa-amide), a novel peptidic urotensin-II receptor antagonist. *Br J Pharmacol*. **137**:449-58.

Behr TM, Nerurkar SS, Nelson AH, Coatney RW, Woods TN, Sulpizio A, Chandra S, Brooks DP, Kumar S, Lee JC, Ohlstein EH, Angermann CE, Adams JL, Sisko J, Sackner-Bernstein JD, and Willette RN (2001) Hypertensive end-organ damage and premature mortality are p38 mitogen- activated protein kinase-dependent in a rat model of cardiac hypertrophy and dysfunction. *Circulation* **104**:1292-1298.

Griffin KA, Churchill PC, Picken M, Webb RC, Kurtz TW, and Bidani AK (2001) Differential salt-sensitivity in the pathogenesis of renal damage in SHR and stroke prone SHR. *Am.J.Hypertens.* **14**:311-320.

Hamilton CA, Brosnan MJ, McIntyre M, Graham D, and Dominiczak AF (2001) Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. *Hypertension* **37**:529-534.

Huot J, Houle F, Marceau F, and Landry J (1997) Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ.Res.* **80**:383-392.

Ju H, Nerurkar S, Sauermelch CF, Olzinski AR, Mirabile R, Zimmerman D, Lee JC, Adams J, Sisko J, Berova M, and Willette RN (2002) Sustained activation of p38 mitogen-activated protein kinase contributes to the vascular response to injury. *J.Pharmacol.Exp.Ther.* **301**:15-20.

Kim I, Moon SO, Park SK, Chae SW, and Koh GY (2001) Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-selectin expression. *Circ.Res.* **89**:477-479.

Kinlay S, Libby P, and Ganz P (2001) Endothelial function and coronary artery disease. *Curr.Opin.Lipidol.* **12**:383-389.

Landmesser U and Harrison DG (2001) Oxidative stress and vascular damage in hypertension. *Coron.Artery Dis.* **12**:455-461.

Lenda DM and Boegehold MA (2002a) Effect of a high salt diet on microvascular antioxidant enzymes. *J.Vasc.Res.* **39**:41-50.

Lenda DM and Boegehold MA (2002b) Effect of a high-salt diet on oxidant enzyme activity in skeletal muscle microcirculation. *Am.J.Physiol Heart Circ.Physiol* **282**:H395-H402.

Liu Y, Rusch NJ, and Lombard JH (1999) Loss of endothelium and receptor-mediated dilation in pial arterioles of rats fed a short-term high salt diet. *Hypertension* **33**:686-688.

Ma XL, Gao F, Nelson AH, Lopez BL, Christopher TA, Yue TL, and Barone FC (2001) Oxidative inactivation of nitric oxide and endothelial dysfunction in stroke-prone spontaneous hypertensive rats. *J.Pharmacol.Exp.Ther.* **298**:879-885.

Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF, and Sluiter W (1997) p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochem.Biophys.Res.Commun.* **230**:44-48.

Raitakari OT and Celermajer DS (2000) Testing for endothelial dysfunction. *Ann.Med.* **32**:293-304.

Read MA, Whitley MZ, Gupta S, Pierce JW, Best J, Davis RJ, and Collins T (1997) Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen- activated protein kinase pathways. *J.Biol.Chem.* **272**:2753-2761.

Ross R (1999) Atherosclerosis--an inflammatory disease. *N.Engl.J.Med.* **340**:115-126.

Schiffrin EL (2002) Beyond blood pressure: the endothelium and atherosclerosis progression. *Am.J.Hypertens.* **15**:115S-122S.

Shimokawa H (1999) Primary endothelial dysfunction: atherosclerosis. *J.Mol.Cell Cardiol.* **31**:23-37.

Tamura DY, Moore EE, Johnson JL, Zallen G, Aiboshi J, and Silliman CC (1998) p38 mitogen-activated protein kinase inhibition attenuates intercellular adhesion molecule-1 up-regulation on human pulmonary microvascular endothelial cells. *Surgery* **124**:403-407.

Touyz RM (2003) The role of angiotensin II in regulating vascular structural and functional changes in hypertension. *Curr.Hypertens.Rep.* **5**:155-164.

Touyz RM, He G, El Mabrouk M, and Schiffrin EL (2001) p38 Map kinase regulates vascular smooth muscle cell collagen synthesis by angiotensin II in SHR but not in WKY. *Hypertension* **37**:574-580.

Verma S and Anderson TJ (2002) Fundamentals of endothelial function for the clinical cardiologist. *Circulation* **105**:546-549.

Wilcox CS (2002) Reactive oxygen species: roles in blood pressure and kidney function. *Curr.Hypertens.Rep.* **4**:160-166.

Yamashita T, Taka T, Nojima R, Ohta Y, Seki J, and Yamamoto J (2002) There is no valid evidence presented as to an impaired endothelial NO system in the stroke-prone spontaneously hypertensive rats. *Thromb.Res.* **105**:507-511.

Zhu Y, Liao H, Wang N, Ma KS, Verna LK, Shyy JY, Chien S, and Stemerman MB (2001) LDL-activated p38 in endothelial cells is mediated by Ras. *Arterioscler.Thromb.Vasc.Biol.* **21**:1159-1164.

Legends:

Figure 1. Chemical structure and kinase activity profiles of p38 MAPK inhibitors used in the present study: SB-239063AN (1-(1,3-dihydroxyprop-2-yl)-4-(4-fluorophenyl)-5-[2-phenoxypyrimidin-4-yl]imidazole and SB-239063 (trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl methoxypyridimidin-4-yl)imidazole).

Figure 2. Temporal activation of p38 MAPK in cultured HUVECs induced by TNF-α and upregulation of phospho-p 38 (P-p38) MAPK in aortae from SHRSP rats. A: A representative Western blot showing specific bands of P-p38 MAPK and p38 MAPK expression in HUVECs after stimulation with TNF-α (1 ng/ml) for 0-60 min. Immunohisthochemical staining of P-p38 MAPK in aorta from normotensive (NT; B; n=5) and SHRSP (C, n=5) rats. P-p38 MAPK immunoreactivity (brown staining as indicated by arrows) was observed only in SHRSP endothelium, outer media and adventitia (Fig. 2B).

Figure 3. Adhesion molecules expression in HUVECs is induced by LPS (100 ng/ml) and TNF-α (1 ng/ml) and attenuated by a p38 MAPK inhibitor, SB-239063AN. Panel A is a representative Western blot showing specific bands for ICAM-1 and PECAM-1 expression in HUVECs induced by LPS (100 ng/ml). SB-239063AN dose-dependently inhibits ICAM-1 expression induced by LPS (A) and TNF-α (B) stimulation in HUVECs.

Figure 4. The time-course of endothelial dysfunction was evaluated by *ex vivo* vascular reactivity in aortae from age matched normal diet (ND-SHRSP), salt/fat diet (SFD-SHRSP) and ND-WKY rats (11-12 weeks of age at entry). Endothelial-dependent vasorelaxation induced by carbachol was compared at 0, 4 and 8 weeks of study. All values are mean ± SEM Significant differences in the maximum response were determined by ANOVA and Bonferroni post hoc test (*$p<0.05$ vs. week 0 SHRSP). N=6 per group at each time-point.

Figure 5. Time course of albuminuria was compared in SHRSPs maintained on a salt-fat diet (SFD-SHRSP) and a normal chow diet (ND-SHRSP). Based on weekly determination of 24-hour urine samples, renal dysfunction was evident within 4-5 weeks in the SFD group. All values are mean ± SEM (*$p<0.05$ vs. ND-SHRSP). N=5-6 animals per group at each time-point.

Figure 6. SB-239063AN treatment enhanced survival in a delayed treatment protocol. SFD SHRSPs (n=52) were placed on a SFD until approximately 10% mortality (n=6) was observed (week 6). At 6 weeks, 18 of the SFD-SHRSPs were randomly chosen to receive the SFD containing SB-239063AN (1500 ppm in SFD *ad libitum*) and the remainder (n=28) continued to receive the SFD (controls). A group of SHRSPs (n=10) received a normal chow diet. Chi-square analysis was performed at each time interval (*$p<0.05$ *vs*. SFD-SHRSP).

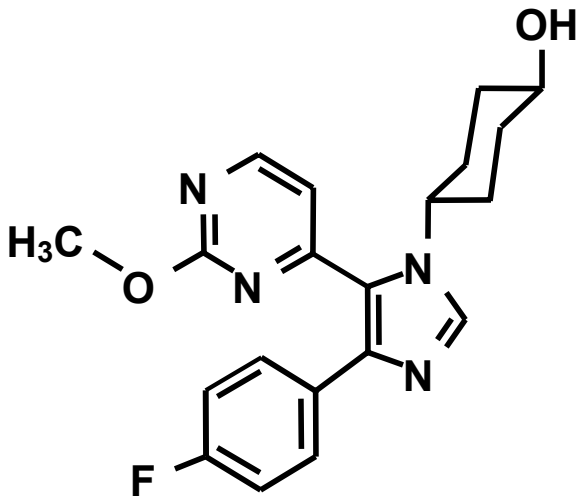
Figure 7. Delayed chronic oral SB-239063AN treatment (12 weeks) restores endothelium-dependent vasorelaxation in SHRSPs receiving the SFD. The study design was as described in Fig 7, i.e. SB-239063AN treatment was begun after 6 weeks. Responses to norepinephrine are expressed as percent of response to 60mM KCl and responses to carbachol and SNP are expressed as percent reversal of norepinephrine contraction. All values are mean ± SEM (*p<0.05 *vs*. SFD-SHRSP).

Figure 8. Blood Pressure, monitored by telemetry, was compared in age-matched SHRSP rats receiving normal diet chow (ND), salt/fat diet (SFD) or a SFD + SB-239063 (1200 ppm in diet). Mean arterial blood pressure (MAP) values in mmHg represent the average obtained each week over a 48-hour period at 5-min intervals. All values are the mean ± SEM (*p<0.05 vs. SFD-SHRSP).

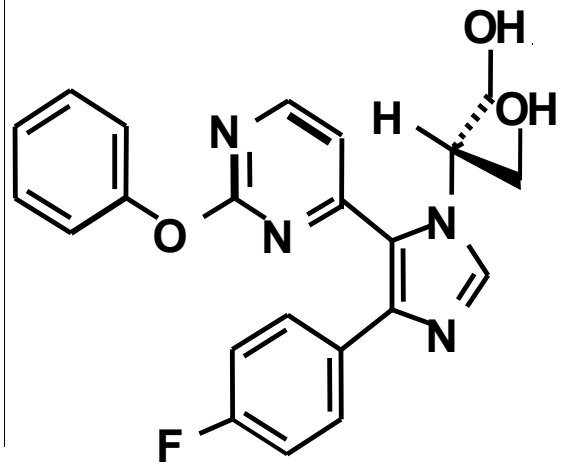
Table 1: Effects of delayed chronic oral SB-281832 treatment on vasoactivity in SHRSPs

Group	*Norepinephrine*		*Carbachol*		*Sodium nitroprusside*	
	EC_{50} *(nM)*	R_{max} *(% response to 60mM KCl)*	EC_{50} *(nM)*	R_{max} *(% reversal of NE-induced tone)*	EC_{50} *(nM)*	R_{max} *(% reversal of NE-induced tone)*
Normal diet control (*n*=8)	11.1 ± 1.1	108.0 ± 2.7	$^{\#\#}$352 ± 57	$^{\#\#}$85.1 ± 2.8	$^{\#\#\#}$4.2 ± 1.0	100
Salt / Fat diet control (*n*=10)	9.2 ± 1.3	101.2 ± 3.1	††819 ± 112	††67.5 ± 3.1	†††11.8 ± 1.3	100
SB-281832 (1500ppm; *n*=7)	12.5 ± 0.7	††93.2 ± 2.1	$^{\#\#}$318 ± 60	$^{\#\#}$85.1 ± 3.9	†8.5 ± 0.3	100

All values are expressed as mean±SEM and *n* represents the number of animals studied. Statistical comparisons were performed using one-way ANOVA analysis with a Tukey-Kramer multiple comparisons post-test: $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, and $^{\dagger\dagger\dagger}P<0.001$ vs. normal diet control and $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. salt/fat diet control.



SB-239063, MW =368.4



SB-239063AN, MW =406.4

Fig. 1

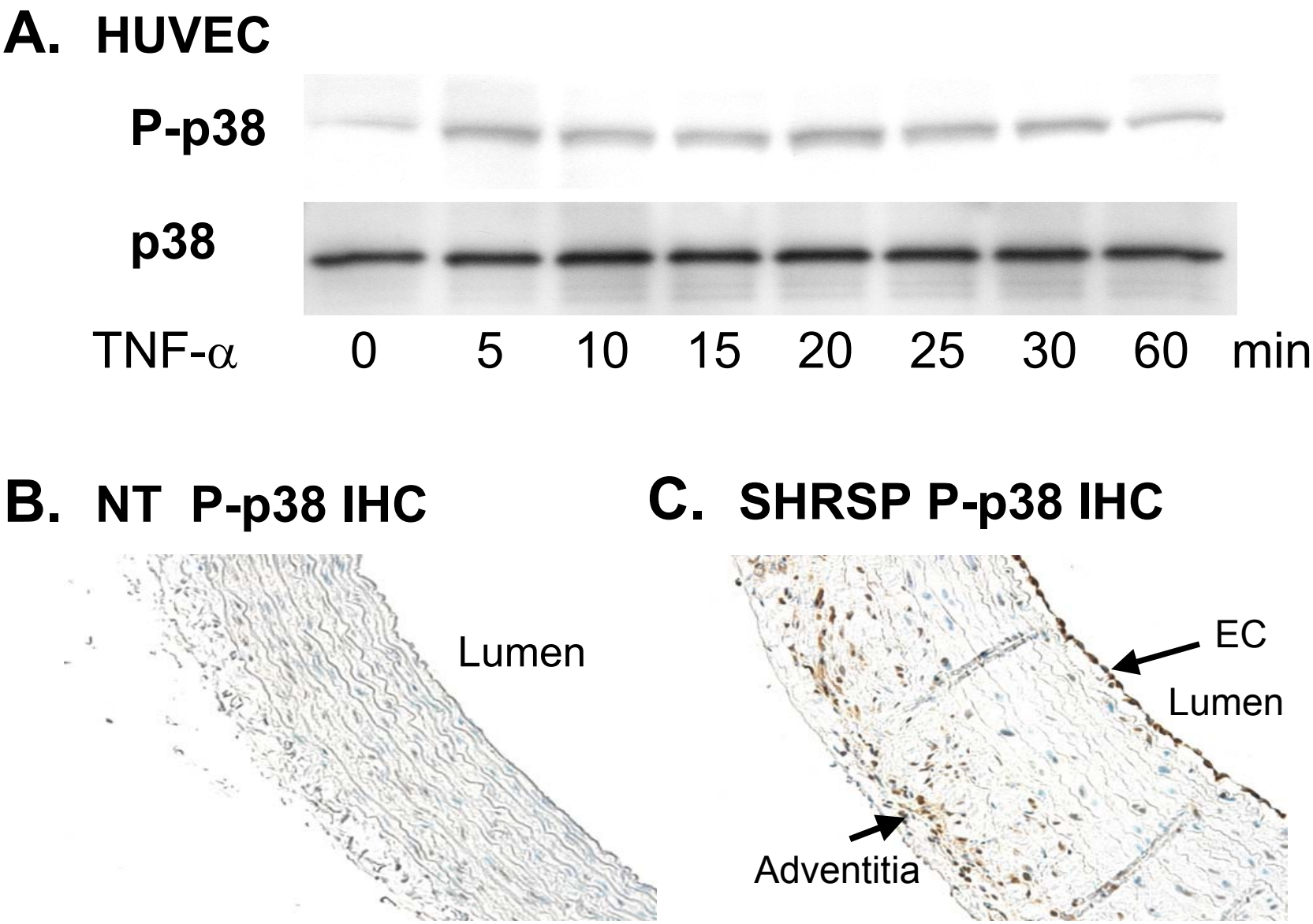
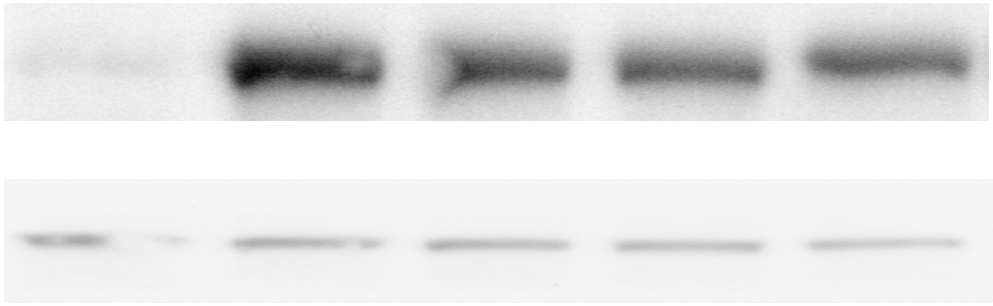

A. HUVEC
P-p38
p38
TNF-α 0 5 10 15 20 25 30 60 min
B. NT P-p38 IHC
Lumen
C. SHRSP P-p38 IHC
EC
Lumen
Adventitia

Fig. 2

A.



B.

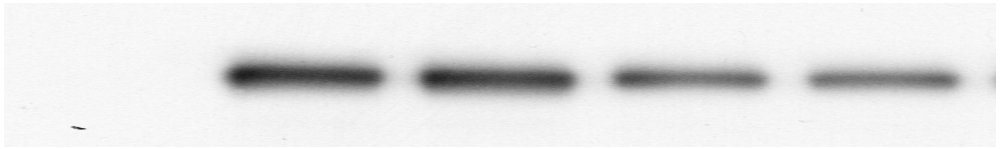


Fig. 3

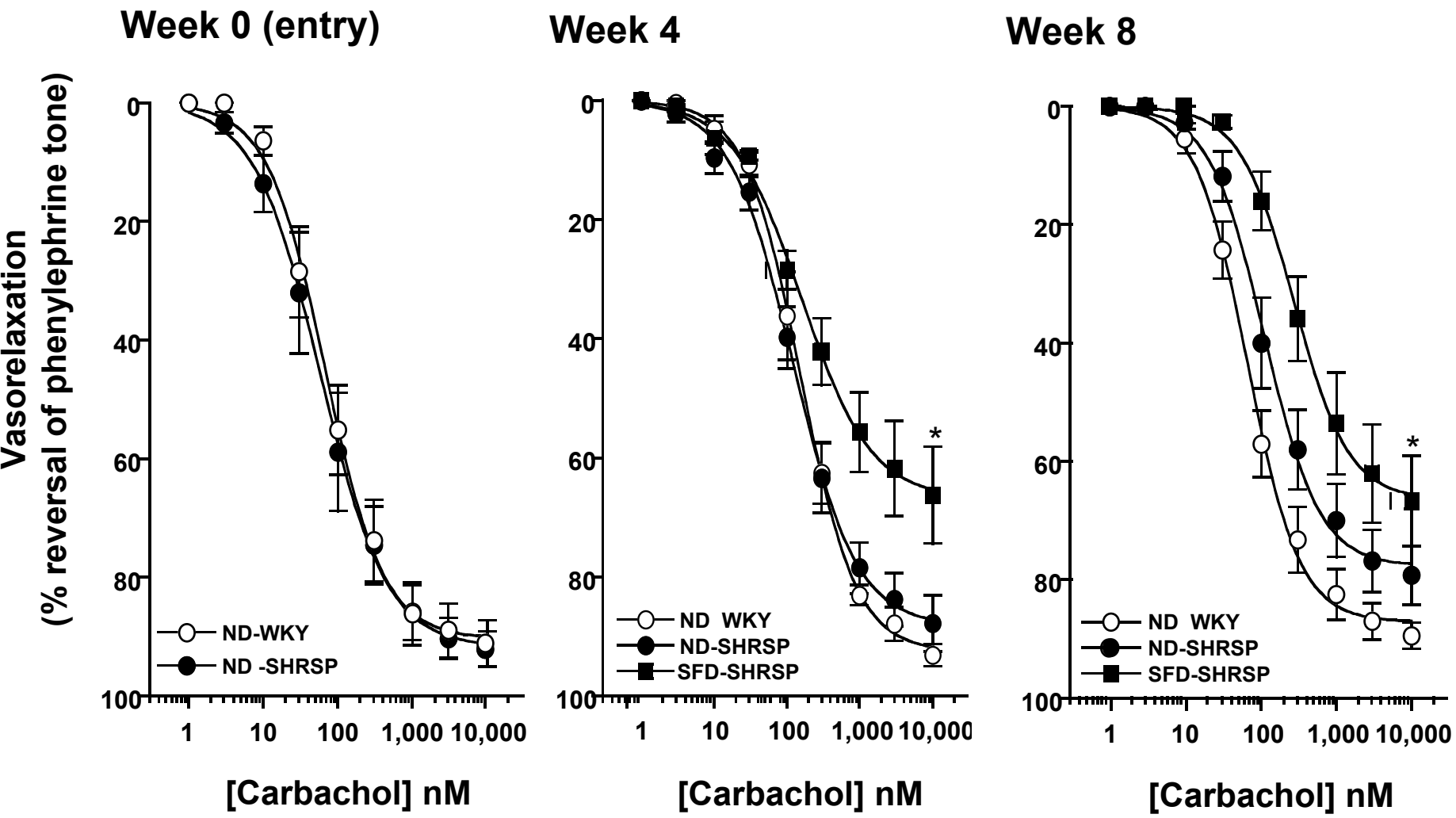

Week 0 (entry)
Week 4
Week 8
Vasorelaxation
(% reversal of phenylephrine tone)
0
20
40
60
80
100
1
10
100
1,000
10,000
[Carbachol] nM
ND-WKY
ND -SHRSP
ND WKY
ND-SHRSP
SFD-SHRSP
*

Fig. 4

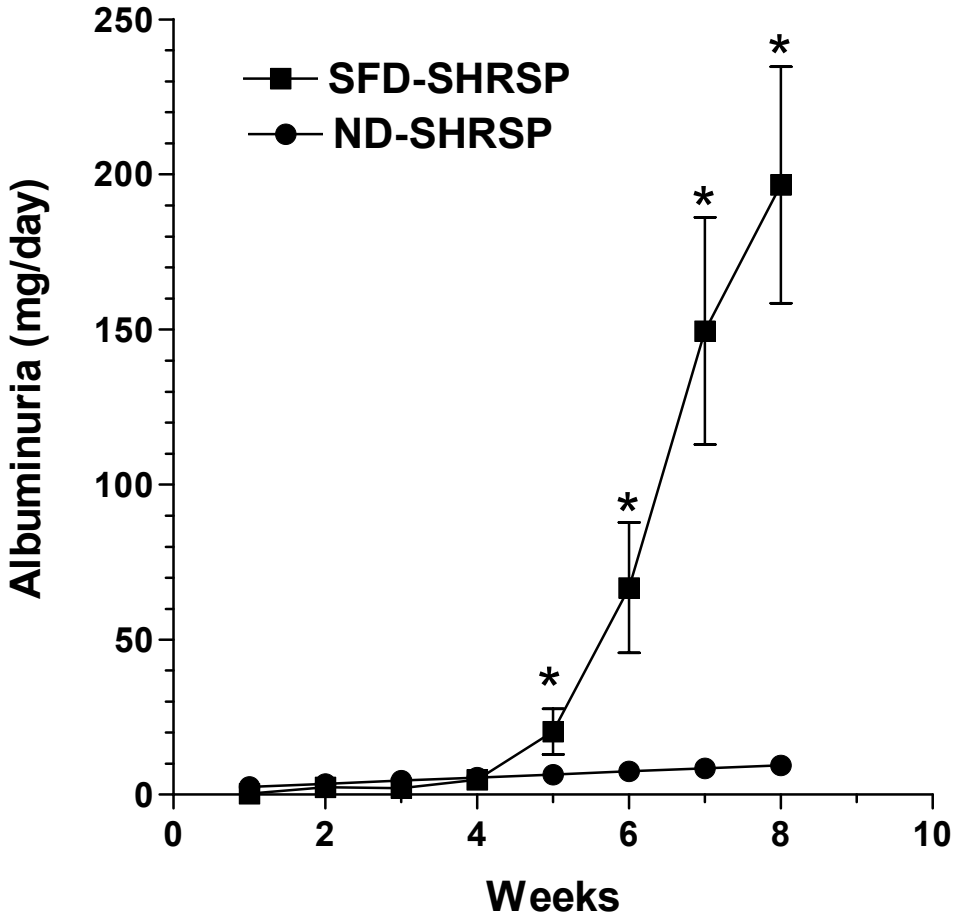

SFD-SHRSP
ND-SHRSP
Albuminuria (mg/day)
Weeks
250
200
150
100
50
0
0
2
4
6
8
10
*
*
*
*

Fig. 5

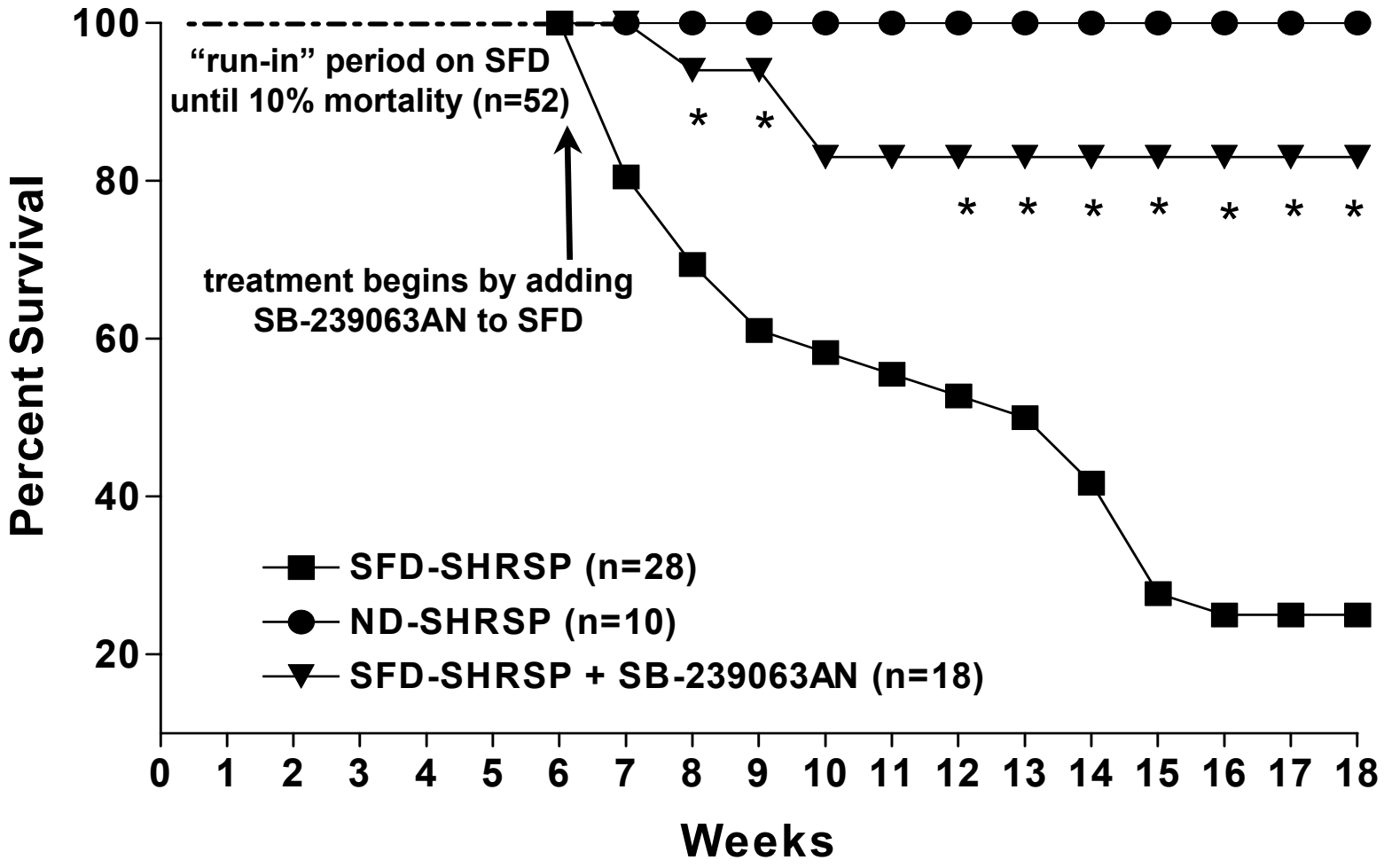

100
80
60
40
20
Percent Survival
"run-in" period on SFD
until 10% mortality (n=52)
treatment begins by adding
SB-239063AN to SFD
SFD-SHRSP (n=28)
ND-SHRSP (n=10)
SFD-SHRSP + SB-239063AN (n=18)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Weeks

Fig. 6

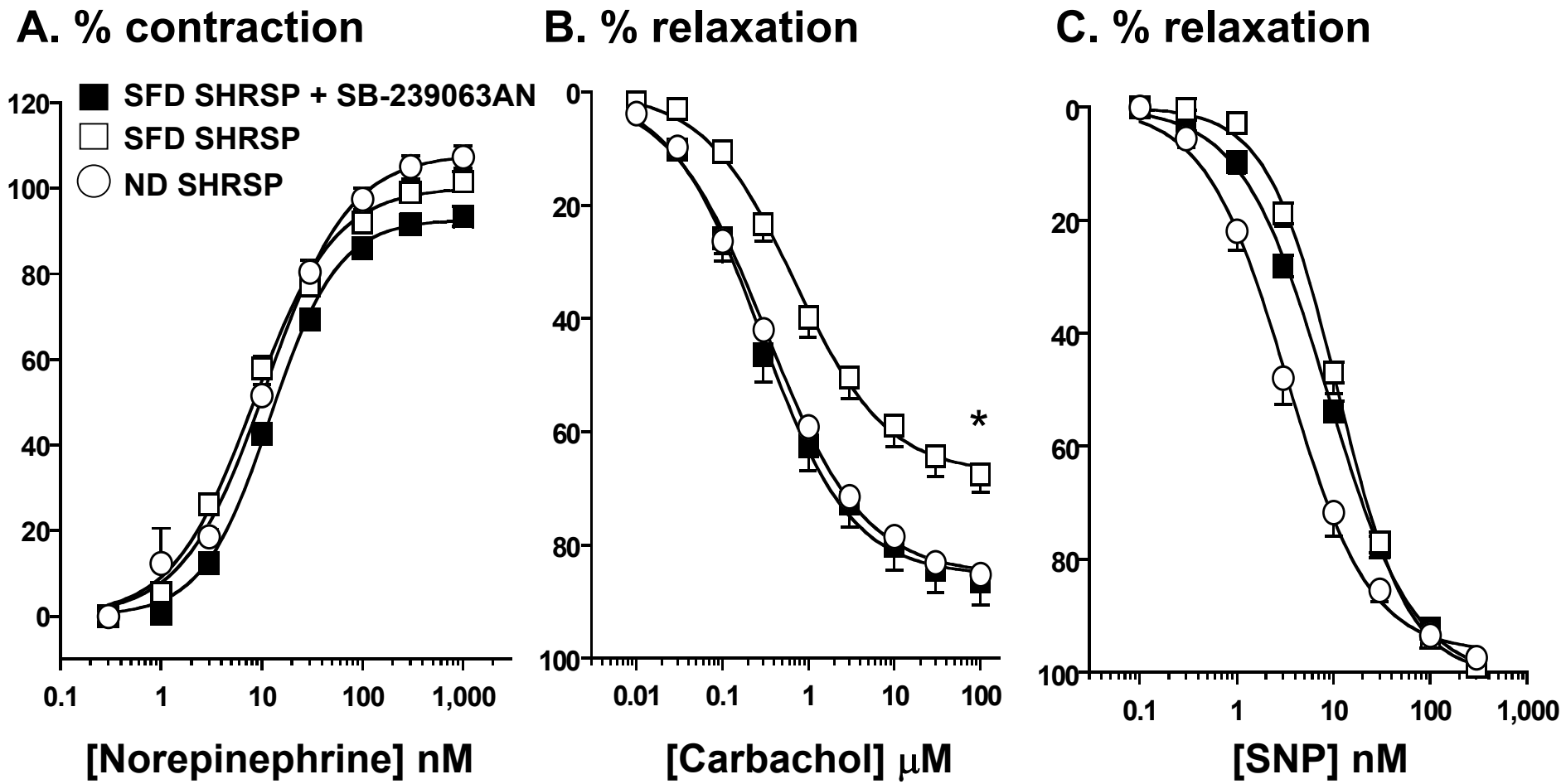

A. % contraction
B. % relaxation
C. % relaxation
SFD SHRSP + SB-239063AN
SFD SHRSP
ND SHRSP
120
100
80
60
40
20
0
0.1
1
10
100
1,000
[Norepinephrine] nM
0
20
40
60
80
100
0.01
0.1
1
10
100
*
[Carbachol] μM
0
20
40
60
80
100
0.1
1
10
100
1,000
[SNP] nM

Fig. 7

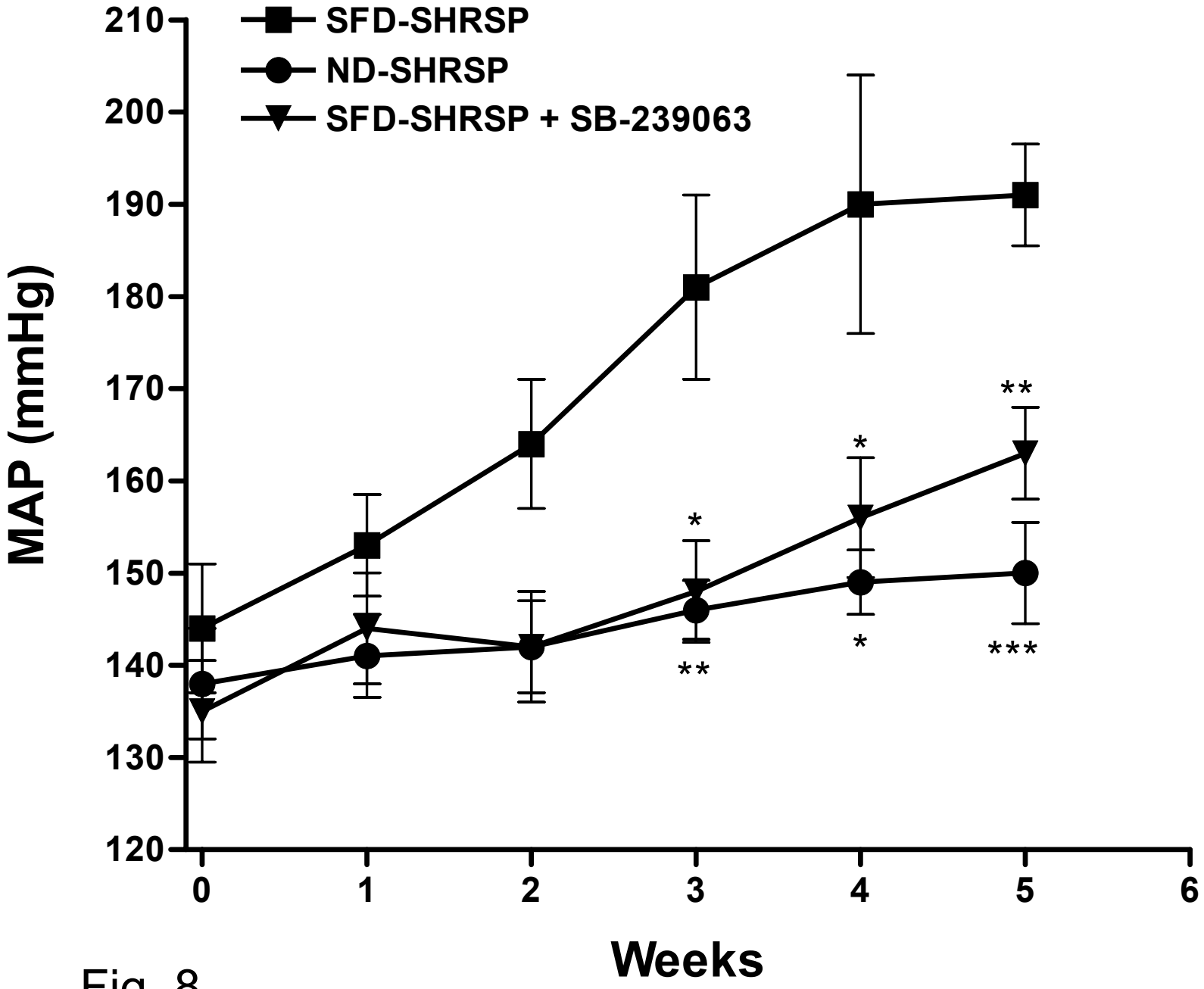

SFD-SHRSP
ND-SHRSP
SFD-SHRSP + SB-239063
MAP (mmHg)
210
200
190
180
170
160
150
140
130
120
0
1
2
3
4
5
6
Weeks
*
**
*
**
*
***

Fig. 8