TERUTROBAN, A TP RECEPTOR ANTAGONIST, INCREASES SURVIVAL IN STROKE-PRONE RATS BY PREVENTING SYSTEMIC INFLAMMATION AND ENDOTHELIAL DYSFUNCTION. COMPARISON WITH ASPIRIN AND ROSUVASTATIN.

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Running title: Terutroban increases survival in stroke-prone rats

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Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; MRI, magnetic resonance imaging; TPr, thromboxane/prostaglandin endoperoxide receptor; ASA, aspirin; RSV, rosuvastatin; eNOS, Endothelial Nitric Oxide Synthase; TXB2, Thromboxane B2; IL1beta, Interleukin 1, beta; CRP, C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1

Recommended section: Cardiovascular
Abstract

This study investigated the efficacy of terutroban, a specific thromboxane/prostaglandin endoperoxide receptor (TPr) antagonist, on stroke incidence in spontaneously hypertensive stroke-prone rats (SHRSP). The effects of terutroban were compared to those of aspirin, another anti-platelet agent, and rosuvastatin, known to exert end-organ protection in SHRSP. Salt-loaded male SHRSP were treated orally once a day with vehicle, terutroban (30 mg/kg/day), aspirin (60 mg/kg/day) or rosuvastatin (10 mg/kg/day). Compared with vehicle, and regardless of any effect on blood pressure or serum TXB2 levels, terutroban significantly increased survival (p<0.001) as a consequence of a delayed brain lesions occurrence monitored by magnetic resonance imaging (MRI) (p<0.001), and a delayed increase of proteinuria (p<0.001). Terutroban decreased cerebral mRNA transcription of IL-1beta, TGF-beta and MCP-1 after 6 weeks of dietary treatment. Terutroban also prevented the accumulation of urinary acute-phase proteins at high molecular weight (HMW), identified as markers of systemic inflammation, and assessed longitudinally by one-dimensional electrophoresis. Terutroban has also protective effects on the vasculature as suggested by the preservation of endothelial function and endothelial nitric oxide synthase (eNOS) expression in isolated carotid arteries. These effects are similar to those obtained with rosuvastatin, and superior to those of aspirin. Terutroban increases survival in SHRSP by reducing systemic inflammation as well as preserving endothelial function. These data support clinical development of terutroban in the prevention of cerebrovascular and cardiovascular complications of atherothrombosis.
Introduction

Several clinical and experimental studies (Widlansky et al., 2003; Huang and Vita, 2006) support the hypothesis that endothelial dysfunction and systemic inflammation play key roles in the pathogenesis of vascular diseases, including myocardial and brain ischemia. Human studies have demonstrated positive association between systemic inflammation induced by endotoxin infusion and marked endothelial dysfunction as well as impaired responses to vasoactive compounds (Pleiner et al., 2004). An analysis of the Framingham Heart Study Offspring cohort found that serum CRP, IL-6 and sICAM-1 levels inversely correlated with brachial artery flow-mediated dilation and reactive hyperemia in the forearm, although this relationship was weakened after adjusting for traditional risk factors (Vita et al., 2004).

Spontaneously hypertensive stroke-prone rats (SHRSP) develop hypertension and proteinuria and die after the onset of cerebrovascular damage, which is invariably preceded by systemic inflammation and endothelial dysfunction (Sironi et al., 2001; Ballerio et al., 2007). Notably, systemic inflammation is characterized by an accumulation - in serum and urine - of acute-phase high molecular weight (HMW) proteins such as thiostatin, the most common marker of inflammation in rat (Sironi et al., 2001). In SHRSP, brain lesions have a vasogenic origin due to the blood-brain barrier impairment (Guerrini et al., 2002). Therefore, this model is particularly suited to reveal cerebrovascular benefits of drugs acting on the inflammatory cascade and/or endothelial dysfunction.

The purpose of this study was to evaluate in SHRSP the effects of terutroban, a highly selective and long-acting TP receptor (TPr) antagonist with antithrombotic, antivasoconstrictive and anti-inflammatory/antiatherosclerotic properties (Cimetière et al., 1998). Previous experimental studies have demonstrated that terutroban prevents vascular wall proliferation and atherogenesis (Cheng et al., 2002; Worth et al., 2005; Viles-Gonzalez et al., 2005), increases anti-oxidant enzymes like glutathione peroxidase (Sebekova et al.,
2007) and has anti-inflammatory actions in vitro and in vivo (decreased macrophage infiltration and ICAM-1) (Cayatte et al., 2000). Terutroban also improved endothelial function in patients with coronary artery disease treated with aspirin (Belhassen et al., 2003). Terutroban is developed in secondary prevention of cerebrovascular and cardiovascular events in patients with an history of ischemic stroke or transient ischemic attack (Bousser et al., 2009a and 2009b).

In this study, the optimally effective dose of terutroban established in previous works was compared to those of aspirin (ASA) and rosuvastatin (RSV) to provide comparative data on end-organ protection and anti-inflammation in SHRSP (Sironi et al., 2005).
Methods

Animals and protocol

Male SHRSP aged 4-5 weeks were obtained from Charles River, Italy (Calco, Lecco, Italy) and were cared for in accordance with our Institution’s guidelines. Fifty-two SHRSP switched to the Japanese permissive low-potassium, low-protein and high-sodium diet (Japanese permissive diet, JPD; Laboratorio Dr. Piccioni, Gessate, Italy: 18.7% protein, 0.63% potassium, 0.37% sodium) plus 1% NaCl in drinking water, were randomly divided into four groups (n=13 each group) and treated orally (gavage) with vehicle (1% hydroxy-ethyl cellulose), terutroban (S 18886) 30 mg/kg/day, ASA 60 mg/kg/day, or RSV 10 mg/kg/day. The dose of terutroban (Servier, France) and rosuvastatin (a kind gift from Astra Zeneca, UK) were chosen on the basis of previous studies performed in the lab (Sironi et al., 2005; Nobili et al., 2006; Gianella et al., 2007). ASA (Sigma, St Louis, Mo) dosage was chosen on the basis of published studies (Qiu et al., 2003; Knight and Johns, 2005).

Baseline measurements were made before the onset of the diet. Systolic arterial blood pressure measured by means of tail-cuff plethysmography (PB Recorder 8006, Ugo Basile, Varese, Italy) and weight were evaluated weekly, then rats were individually housed in metabolic cages for 24 hours to collect urine for proteinuria determinations (Bradford’s method) and proteomic studies. Blood was drawn every week from the tail vein; serum was obtained and stored at -20°C until analysed. All rats underwent weekly magnetic resonance imaging (MRI) until 24-h proteinuria reached 100 mg/day, and then every two days until cerebrovascular damage was detected. After six weeks (i.e when the vehicle-treated rats developed brain lesions) five animals from each group were sacrificed to collect the brain as well as the carotid artery.

MRI evaluation of brain damage
The rats were anesthetised with 1.5% isofluorane (Merial, Toulouse, France) in 70% N₂/30% O₂, and placed inside a Bruker AvanceII 4.7T with a micro-imaging accessory. After a scout image, sixteen contiguous 1 mm thick slices were analyzed caudally to the olfactory bulb using a field of view (FOV) of 4 x 4 cm², and a turbo spin echo sequence with 16 echoes per excitation, 10 ms inter-echo time, 85 ms equivalent echo time, and 4 s repetition time. Eight T2-weighted images of 128 x 128 pixels (zero-filled to 256x256) were averaged in 8’30”. The occurrence of lesions was defined as the presence of areas of high signal intensity on T2-weighted images.

Proteinuria studies

One-dimensional electrophoresis (1-DE) of urine proteins (50 μg) was run in the presence of SDS without sample reduction in a discontinuous buffer system on 4-12% polyacrylamide gels stained with Colloidal Blue. Densitometry was performed using Quantity One version 4.5.2 (Biorad, Hercules, CA) to evaluate the percentage of low molecular weight (LMW) and high molecular weight (HMW) proteins density.

Determination of TXB₂ and 11-dehydro-TXB₂

The serum levels of TXB₂ and urinary levels of 11-dehydro-TXB₂ were measured using commercial kits (Cayman Chemical Co., Ann Arbor, MI).

Brain tissue expression of inflammatory markers

After six weeks of dietary treatment, five animals from each group were sacrificed to collect the brains. Total RNA was prepared by means of guanidium thiocyanate denaturation from forebrain homogenates. Reverse transcription polymerase chain reaction (RT-PCR) was used
to evaluate the expression of IL-1beta, TGF-beta, and MCP-1. All the reagents used were purchased from Invitrogen (Carlsbad, CA)

Expression of eNOS

eNOS expression was evaluated by RT-PCR on carotid artery homogenates from animals sacrificed after six weeks of treatment.

Endothelial dysfunction

Isolated carotid artery rings (3 mm) were suspended in an individual organ bath filled with Krebs solution and their vascular reactivity was evaluated as previously described (Ballerio et al., 2007). Indomethacin (10^{-5} \text{ mol/L}; Chiesi Farmaceutici S.p.A., Parma) was added to Krebs solutions in order to inhibit prostanoid synthesis. Arteries were challenged with KCl (100 mM/L) to check the viability of tissues; vessels not responding to KCl were discarded. Vascular smooth muscle function was determined by cumulative addition of L-phenylephrine (L-Phe; Sigma-Aldrich, St. Louis, MO, USA) (10^{-9}–10^{-5} \text{ mol/L}), the contraction response being expressed as the percentage of KCl response. Subsequently, the rings were constricted to their individual EC_{80} value for L-Phe, and maximum smooth muscle relaxation to sodium nitroprusside was determined (SNP; Sigma-Aldrich, St. Louis, MO, USA) (10^{-10}–3\times10^{-6} \text{ mol/L}). After wash-out, the rings were constricted to their individual EC_{80} value for L-Phe, and endothelium-dependent relaxation in response to acetylcholine (Ach; Sigma-Aldrich, St. Louis, MO, USA) (10^{-9}–10^{-5} \text{ mol/L}) was studied both in the absence or presence of L-NAME 10^{-4} \text{ mol/L}. The relaxation responses were expressed as the percentage of L-Phe-induced contraction.

Statistics
Between-group differences were computed by means of analysis of variance (ANOVA) followed by an appropriate post hoc test; the between-group differences in proteinuria, LMW and HMW protein density were computed by means of ANOVA for repeated measurements over time followed by Tukey’s post hoc test. An unpaired t test was used to compare baseline and vehicle-treated group data. Concentration–response curves were statistically analysed using ANOVA followed by Tukey’s or Tamhane’s T2 post hoc test. Sensitivity to the antagonists (pD2) was expressed as the negative logarithm of half-maximal effective concentration (EC50) calculated from individual curves. Results are expressed as means ± S.D. P<0.05 was considered statistically significant.
Results

Physiological parameters and survival of SHRSP

Body weight increased similarly in all experimental groups. The severe hypertension that developed was not affected by any of the drug treatments (Fig. 1A). Plasma total cholesterol and triglyceride levels (42.38±3.89 and 68.5±8.96 mg/dl respectively at baseline), did not significantly change during the treatment period in any groups. Vehicle-treated animals developed cerebral lesions 42.4±10.8 days after starting salt loading. All treatments significantly delayed the appearance of cerebrovascular damages (Fig. 1B and 1D). However, the delay of occurrence observed under terutroban (87.6±19.2 days; p<0.001) was greater than that induced by aspirin (63.9±9.01 days; p<0.05) and comparable to that observed after RSV (85.6±16.9 days; p<0.001). Comparison of survival clearly shows the effectiveness of all treatments (Fig.1C). Compared to the vehicle group, survival was similarly increased by terutroban and RSV (p<0.001) and this effect was significantly superior to that observed after aspirin treatment (RSV p<0.05; terutroban p< 0.01).

Serum TXB2 and urinary 11-dehydro-TXB2 levels

As expected, serum TXB2 and urinary 11-dehydro-TXB2 levels were significantly decreased by ASA while the levels were not affected by salt loading, nor by RSV or terutroban treatment (Fig. 2A and 2B).

Proteinuria and composition of urinary proteins

The SHRSP receiving vehicle developed progressively a severe proteinuria. After 4.7±1.3 weeks of salt loading, proteinuria was higher than 100 mg/day and increased rapidly and linearly to reach an average of 266±28.9 mg/day after 7 weeks. Treatment with terutroban and RSV delayed significantly the increase in proteinuria (10.5±3.4 weeks, p<0.001, and 9.1±1.9
weeks, p<0.01 vs vehicle respectively) (Fig. 2C), whereas ASA had only a slight effect (6.2±1.2 weeks, n.s.) on this parameter. At the beginning of the experiment (week 1, Fig. 3), the most abundant excreted protein was the major urinary protein (MUP or alpha-2u-globulin) which represents the major protein excreted in urine of healthy male rats (Sironi et al., 2001). MUP, together with other LMW proteins, accounted for about 70% of the total protein content. Protein composition changed over time in the salt-loaded SHRSP, with an accumulation of HMW proteins previously identified as markers of inflammation (Ballerio et al., 2007), and a simultaneous decrease in LMW proteins (Fig. 3A). In the vehicle group, HMW proteins reached 70% of the total protein content 4-5 weeks after the start of dietary treatment (Fig. 3A); in the ASA- and RSV- treated rats, HMW proteins became preponderant after seven and nine weeks respectively (Fig. 3B and 3C). Densitometric analysis of the excreted proteins in the terutroban group showed that the HMW proteins level did not increase to more than 50% of total protein content even after 14-15 weeks of treatment (Fig. 3D).

Brain expression of inflammatory markers

In comparison with vehicle-treated animals, all the drugs markedly reduced the accumulation of IL-1beta, MCP-1 and TGF-beta mRNA in the brain tissues (Fig. 4).

Endothelial dysfunction

The response curves to phenylephrine in carotid artery rings showed significantly reduced contraction in terutroban-treated rats (p<0.05). ASA and RSV treatment also tended to reduce the contractions caused by phenylephrine (n.s.) (Fig. 5A). In rings pre-contracted with phenylephrine, the concentration-response curves to the administration of the NO donor sodium nitroprusside were comparable in all groups (Fig. 5B). The endothelium-dependent
relaxation evoked by acetylcholine was not altered by ASA, but was significantly increased by terutroban and RSV (p<0.01) (Fig. 5C). Incubation with L-NAME abolished the acetylcholine-induced relaxation in all experimental groups (Fig. 5C). There was no difference in sensitivity (pD$_2$) among the experimental groups whatever the experimental condition (Tab. 1).

Carotid eNOS expression

Terutroban and rosuvastatin increased the expression of eNOS mRNA (1.98±0.66, p<0.05 and 1.85±0.40, n.s. vs 1.04±0.37 AU in vehicle group respectively), while aspirin did not have any effect (1.31±0.6 AU).

Discussion

In this study, we investigated the effects of terutroban, a TP receptor antagonist, on the pathological events that spontaneously develop in SHRSP. The effects induced by terutroban were compared with those of aspirin, a cyclo-oxygenase inhibitor, and rosuvastatin, a statin which demonstrated beneficial effects in this model (Sironi et al., 2005).

In salt-loaded SHRSP, terutroban delayed the occurrence of spontaneous brain lesions and consequently increased the survival, regardless of any effect on blood pressure or on serum TXB$_2$ levels. Terutroban was more effective in brain damage protection than aspirin, and had similar effects as rosuvastatin. In a previous study, a beneficial effect was also observed when terutroban was administered three weeks after the start of dietary treatment, thus indicating that terutroban can also reverse ongoing pathological events in salt-loaded SHRSP (Gelosa et al., 2007).

Mechanisms that could contribute to the beneficial effect of TP receptor blockade on stroke prevention in this model, are an attenuation of the systemic inflammation that invariably
precedes the occurrence of cerebrovascular events, as well as a preservation of vascular reactivity.

One of the features of the systemic inflammation that develops in SHRSP is the progressive urinary accumulation of HMW proteins (Sironi et al., 2001), which reached 70% of total urinary protein excretion after 4-5 weeks of dietary treatment. Previously published data have shown that these proteins, solved with 2-dimensional electrophoresis, consist in markers of inflammatory response such as kallikrein-binding protein, transthyretin, albumin, alpha-1-antitrypsin and thiostatin. These proteins are markers of an inflammatory response and their accumulation in body fluids invariably precedes the occurrence of brain abnormalities (Sironi et al., 2001). Terutroban significantly attenuated this accumulation with a level of HMW proteins less than 50%, confirming the major anti-inflammatory activity of terutroban at the systemic level, which is superior to that of rosuvastatin and aspirin treatments. Terutroban prevents also the accumulation of IL-1beta, MCP-1 and TGF-beta mRNA in brain tissue. These data are consistent with previous in vitro and in vivo experiments showing that TPr stimulation is significantly involved in inflammatory processes and, that blockade of this receptor with terutroban reduces inflammatory markers in various experimental models (Cayatte et al., 2000; Zuccollo et al.2005; Xu et al., 2006). Ishizuka et al. have also reported that TP receptor blockade with ramatroban (BayU3405) suppressed the expression of inflammatory mediators (particularly MCP-1) in stimulated vascular endothelial cells (Ishizuka et al., 2000), and that the TPr antagonist ONO-8809 contributed to cerebral protection in salt-loaded SHRSP by reducing macrophage accumulation and MMP-9 activity (Ishizuka et al., 2007). Similarly to terutroban, rosuvastatin and aspirin attenuated significantly brain inflammation. We have previously reported that rosuvastatin prevented inflammatory processes associated with cerebrovascular disease, and this independently of changes in plasma lipid levels (Sironi et al., 2005). Aspirin had also demonstrated numerous
pharmacological activities including anti-oxidant and anti-inflammatory effects. Recently, Ishizuka et al. (Ishizuka et al., 2008) revealed that aspirin may inhibit the cerebrovascular inflammation in SHRSP through anti-oxidative properties.

In addition to its anti-inflammatory activity, terutroban significantly preserves vascular reactivity to a greater extent than aspirin and rosuvastatin. Analysis of the concentration-response curves of carotid artery rings showed that terutroban reduces the contraction elicited by phenylephrine, without affecting pD2, thus indicating that the adrenergic receptor signal transduction mechanisms are not altered. Aspirin and rosuvastatin have beneficial but lesser effect on this parameter.

The endothelium-dependent relaxation induced by acetylcholine was significantly improved by terutroban and rosuvastatin in comparison with vehicle and aspirin. This is consistent with clinical data showing that a single oral dose of terutroban significantly improved endothelium-dependent vasodilation in the peripheral arteries of patients with coronary artery disease treated with aspirin, thus strengthening the hypothesis that terutroban has additional therapeutic benefits such as (1) allowing the production of vasodilating prostanoids (e.g. prostacyclin), which is impaired by COX inhibition and (2) inhibiting the production of vasoconstrictor prostanoids other than TXA2 (Cayatte et al., 2000). Moreover, the improvement of endothelium-dependent relaxation was inhibited after incubation with L-NAME, suggesting a partial restoration of NO release or synthesis by terutroban. This hypothesis is strengthened by the increased expression of eNOS mRNA in the carotid arteries of animals treated with terutroban, whereas expression of eNOS mRNA was not changed significantly in animals treated by aspirin. This is in agreement with previous results showing an increase in eNOS expression in aorta of diabetic mice treated by terutroban (Zuccollo et al., 2005).
Benefits on survival induced by terutroban were independent of modifications in TXB2 levels, which remained unchanged after terutroban administration, contrary to aspirin, which almost suppressed the production of TXA2 (as reflected by a reduction of its serum metabolite TXB2) but with an effect on survival that was significantly inferior to that obtained with terutroban. The greater effect of TPr antagonism with terutroban on brain protection could be attributed to a greater effect in inflammation processes at systemic level, and is probably due to ligands other than TXA2 and prostaglandins-endoperoxides (PGG2 and PGH2). It was beyond the scope of our study to identify the eicosanoids potentially responsible for activating the inflammatory cascade involved in end-organ damage in SHRSP, but possible candidates are the isoprostanes, produced from arachidonic acid by non enzymatic oxidation, and whose formation is not influenced by COX inhibitors. This hypothesis is corroborated by the results obtained by Ishizuka et al. (Ishizuka et al., 2007) who suggested that cerebrovascular inflammation in salt-loaded SHRSP may be due to TP receptor stimulation by 8-iso- PGF2α.

In this study, the effect of terutroban was similar to that of rosuvastatin, an effective drug in preventing end-organ damage in a model of SHRSP (Sironi et al., 2005). Terutroban increased survival to a greater extent than aspirin, probably due to its greater effects on systemic inflammation and endothelial dysfunction.

The benefits of terutroban on survival and pathological events occurring in SHRSP may therefore be attributed to its anti-inflammatory activity, along with the improvement of endothelial function.

Controlling inflammation and preserving endothelial function are key factors for preventing the development of the spontaneous brain damage occurring in SHRSP. In addition to platelet aggregation inhibition, terutroban also offers the therapeutic benefit of anti-inflammatory and vascular protective properties, which support its clinical development in the prevention of cerebrovascular and cardiovascular complications of atherothrombosis.
Acknowledgements

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References


Footnotes

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Legends for figures

Figure 1. Effects of vehicle (squares, n=8), ASA (triangles, n=8), RSV (stars, n=8) and terutroban (circles, n=8) on: systolic blood pressure (A), appearance of brain damage (B) and survival (C). Panel B: *** p< 0.001, * p< 0.05 vs vehicle; † p< 0.05 vs terutroban and RSV. Panel C: * p< 0.05, ** p< 0.001 versus vehicle group. Panel D shows representative MRI images from healthy (left) and damaged (right) brain; the lesion(s) visualized by T2W-MRI appears as a hyperintense area, pointed out by the arrows.

Figure 2. Panels A and B: effects of vehicle, ASA, RSV and terutroban-treatment on serum TXB2 and urinary 11-dehydro-TXB2 levels (n=5 each group); *** p<0.001 vs vehicle, terutroban and RSV, † p<0.05 vs. vehicle and RSV. Panel C: delay in the appearance of proteinuria >100 mg/day; ***p<0.001, ** p<0.01 vs vehicle, †† p<0.01 vs terutroban.

Figure 3. Analysis of urinary proteins by 1-DE. The panels on the left show the results of the densitometric analyses expressed as the percentages of high (HMW) and low (LMW) molecular weight proteins over time in rats treated with vehicle (A), ASA (B), RSV (C) or terutroban (D); n=6 for each group. The panels on the right show representative images of gels for each condition. *p<0.05 vs densitometric HMW value at week 1; § p<0.05 vs densitometric LMW value at week 1.

Figure 4. RT-PCR analysis of inflammatory mediators mRNA transcription in the forebrain of rats treated with vehicle, terutroban, ASA or RSV (n=5 for each condition) and sacrificed after six weeks of dietary treatment. The bars show the densitometry of the PCR bands normalised to the corresponding GAPDH signals. *** p<0.001 and ** p<0.01 vs vehicle.
Figure 5. Effects of the in vivo pharmacological treatments on the cumulative concentration/response curves of carotid rings from SHRSP. Panel A: Phenylephrine-induced contraction: * p<0.05 terutroban vs vehicle group. Panel B: Sodium nitroprusside-induced relaxation. Panel C: Acetylcholine-induced relaxation before and after incubation with L-NAME 10^{-4} M: ** p<0.01 terutroban vs vehicle group and †† p<0.01 RSV vs vehicle group. Data were collected from five rats for each experimental condition.
Table 1. Sensitivity to the antagonists (pD2) expressed as the negative logarithm of EC$_{50}$

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Mean values ± standard deviation
Figure 1

A. Blood pressure

B. Appearance of brain damage

C. Survival

D. Representative MRI images
Figure 2

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TXB$_2$

ng/ml

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B  
Urinary 11-dehydro-TXB$_2$

pg/mg creatinine

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C  
Delay appearance of proteinuria > 100 mg/day

weeks

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Figure 3

A. **Vehicle**

B. **ASA**

C. **RSV**

D. **Terutroban**

Weeks of dietary treatment
Figure 4

**MCP-1**

**IL-1 beta**

**TGF-beta**
Figure 5

A. Contraction (% vs KCl) vs L-phenylephrine (pM)

B. Relaxation (% vs L-Phe) vs Sodium Nitroprusside (pM)

C. Relaxation (% vs L-Phe) vs Acetylcholine (pM)

- Vehicle
- Terutroban
- ASA
- Rosuvastatin
- Vehicle + L-NAME
- Terutroban + L-NAME
- ASA + L-NAME
- Rosuvastatin + L-NAME