JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305

A long-acting and highly selective prostacyclin receptor agonist prodrug, NS-304, ameliorates rat pulmonary hypertension with unique relaxant responses of its active form, MRE-269, on rat pulmonary artery

Keiichi Kuwano, Asami Hashino, Kumiko Noda, Keiji Kosugi and Kenji Kuwabara

Discovery Research Laboratories (Kei.Ku., A.H., K.N., Kei.Ko., Ken.K.), Nippon Shinyaku Co., Ltd., 14 Nishinosho-Monguchi-Cho, Kisshoin, Minami-Ku, Kyoto 601-8550, Japan

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: A selective IP Receptor Agonist in pulmonary Hypertension

Correspondence: Keiichi Kuwano

Discovery Research Laboratories, Nippon Shinyaku Co., Ltd.

Address: 14 Nishinosho-Monguchi-Cho, Kisshoin, Minami-Ku, Kyoto 601-8550, Japan

Phone: +81 (75) 321-9179; Fax: +81 (75) 314-3269

e-mail: k.kuwano@po.nippon-shinyaku.co.jp

Number of text pages: 32

Number of tables: 0

Number of figures: 8

Number of references: 36

Number of words in abstract: 247

Number of words in introduction: 684

Number of words in discussion: 1319

Abbreviations

2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-*N*-(methylsulfonyl)acetamide--

-------NS-304

{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid --------- MRE-269

(2*E*)-3-(3',4'-dichlorobiphenyl-2-yl)-*N*-(2-thienylsulfonyl)acrylamide --------- DBTSA

prostacyclin --------- PGI_2

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

prostacyclin receptor --------- IP receptor

pulmonary hypertension --------- PH

pulmonary arterial hypertension --------- PAH

monocrotaline --------- MCT

ratio of the weight of the right ventricle to that of the left ventricle plus septum ---------

RV/(LV+S)

right ventricular hypertrophy --------- RVH

right ventricular systolic pressure --------- RVSP

heart rate --------- HR

large pulmonary arteries --------- LPA

small pulmonary arteries --------- SPA

LPA with endothelium --------- LPA(+)

LPA without endothelium --------- LPA(-)

SPA without endothelium --------- SPA(-)

inhibition constant --------- K_i

Chinese hamster ovary --------- CHO

Recommended section: Cardiovascular

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

NS-304 is an orally available, long-acting non-prostanoid prostacyclin receptor (IP receptor) agonist prodrug. In a rat model of pulmonary hypertension (PH) induced by monocrotaline (MCT), NS-304 ameliorated vascular endothelial dysfunction, pulmonary arterial wall hypertrophy, right ventricular hypertrophy, and elevated right ventricular systolic pressure, and improved survival. MRE-269, the active form of NS-304, is much more selective for the IP receptor than are the PGI_2 analogs beraprost and iloprost, which also have high affinity for the EP_3 receptor. To investigate the effect of receptor selectivity on vasodilation of the pulmonary artery, we assessed the relaxant response to these IP agonists in rats. MRE-269 induced vasodilation equally in large pulmonary arteries (LPA) and small pulmonary arteries (SPA), whereas beraprost and iloprost induced less vasodilation in SPA than in LPA. An EP_3 agonist, sulprostone, induced SPA and LPA vasoconstriction, and an EP_3 antagonist attenuated the vasoconstriction. Beraprost showed EP_3 agonism and induced LPA and SPA vasoconstriction, while the EP_3 antagonist inhibited this vasoconstriction and enhanced beraprost- and iloprost-induced SPA vasodilation. These findings suggest that the EP_3 agonism of beraprost and iloprost interfered with the SPA vasodilation resulting from their IP receptor agonism. Endothelium removal markedly attenuated the vasodilation induced by beraprost, but not that induced by MRE-269 or iloprost. Moreover, the vasodilation induced by beraprost and iloprost, but not that induced by MRE-269, was more strongly attenuated in LPA from MCT-treated rats than from normal rats. NS-304 is a promising alternative medication for PAH with prospects for good patient compliance.

Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening disorder characterized by a progressive increase in pulmonary arterial pressure and pulmonary vascular resistance, leading ultimately to right ventricular failure and death (Rich et al., 1987; Farber and Loscalzo, 2004). The pathogenesis of PAH consists of at least three processes occurring in small pulmonary arteries: vasoconstriction, vascular remodeling, and thrombosis (Rubin, 1997; Runo and Loyd, 2003).

Prostacyclin (PGI_2) is a potent endogenous vasodilator and inhibitor of platelet aggregation that acts at the PGI_2 receptor (IP receptor) (Namba et al., 1994). PGI_2 contributes to the maintenance of homeostasis and has various other physiological effects in many organ systems (Narumiya et al., 1999). A decrease in PGI_2 production and an imbalance between PGI_2 and thromboxane A_2 has been reported in PAH (Christman et al., 1992; Tuder et al., 1999), and IP receptor knockout results in a worsening of pulmonary hypertension (PH) (Hoshikawa et al., 2001). These findings indicate that the PGI_2-IP system is involved in the progression of PAH. Treatment with IP receptor agonists or transfer of the PGI_2 synthase gene ameliorates PH in rats treated with monocrotaline (MCT) (Miyata et al., 1996; Nagaya et al., 2000).

When epoprostenol (PGI_2) therapy was developed in the 1990s, it improved the outcome of PAH treatment. Long-term treatment with PGI_2 markedly improves exercise tolerance and survival (Barst et al., 1996). However, PGI_2 therapy requires continuous infusion through a central venous catheter because of the very short half-life of PGI_2 (3-5 min). Despite its success in the treatment of PAH in some patients, PGI_2 infusion is still far

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

from an ideal therapy because of its high cost, the discomfort associated with its administration, and the high risk of complications from infection (Humbert et al., 1999; Sitbon et al., 1988). Many kinds of PGI_2 analog have been synthesized to improve on the chemical stability of PGI_2, and some of them, such as beraprost, iloprost, and treprostinil, have been applied to the clinical treatment of PAH (Olschewski et al., 2002; Simonneau et al., 2002). Nevertheless, their duration of action is so short that continuous infusion or frequent administration is still needed.

To overcome the drawbacks of PGI_2 and its analogs related to their short half-lives, we synthesized a novel diphenylpyrazine derivative, NS-304 (2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-*N*-(methylsulfonyl)acetamide). NS-304 is an orally available and long-acting IP receptor agonist prodrug, and its active form, MRE-269 ({4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid), is a highly selective IP receptor agonist (Kuwano et al., 2007). In addition to IP receptor agonists, endothelin receptor antagonists and phosphodiesterase type 5 inhibitors are also effective treatments for PAH as orally available drugs (Rubin et al., 2002; Galiè et al., 2005). Clinical studies have shown beneficial and significant effects of combination therapy with these three classes of drugs (McLaughlin et al., 2006; Mathai et al., 2007). However, an ideal combination therapy cannot be provided at present because there is no orally available and long-lasting drug in the IP receptor agonist class. It is hoped that NS-304 will satisfy this unmet need and allow the development of an ideal combination therapy that includes members of all three types of orally available drug.

The binding affinity of MRE-269 for the human IP receptor is over 130 times its

affinity for other human prostanoid receptors. Most PGI_2 analogs, such as beraprost and iloprost, show poor selectivity for the IP receptor because they also have high affinity for EP receptor subtypes, especially the EP_3 receptor (Kiriyama et al., 1997; Abramovitz et al., 2000; Kuwano et al., 2007). Because EP_3 receptor agonists cause arterial contraction (Qian et al., 1994; Jones et al., 1998), EP_3-receptor-mediated agonism interferes with IP-receptor-mediated vasorelaxation (Jones et al., 1997; Chan et al., 2004). In fact, we have reported that the effect of beraprost on the increase in femoral skin blood flow in rats is inferior to that of the more selective IP receptor agonist prodrug NS-304 (Kuwano et al., 2007).

In the present study, we show that NS-304 ameliorated various pathological processes associated with PH in MCT-treated rats, and that the pharmacological characteristics of its active form, MRE-269, in the vasodilation of pulmonary artery preparations were superior to those of beraprost and iloprost.

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

Materials

NS-304, MRE-269, and the EP_3 receptor antagonist (2*E*)-3-(3',4'-dichlorobiphenyl-2-yl)-*N*-(2-thienylsulfonyl)acrylamide (hereinafter referred to as DBTSA) were synthesized in our laboratory as previously described (Gallant et al., 2002; Kuwano et al., 2007). Beraprost was purchased from Chinoin (Budapest, Hungary), iloprost, $PGF_{2\alpha}$ and sulprostone from Cayman Chemical Company (Ann Arbor, MI), and monocrotaline (MCT) from Sigma (St. Louis, MO).

Animals

Male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) were housed in cages under a 12-h light-dark cycle and allowed free access to pellet chow and tap water. All animal procedures were approved by the Committee for the Institutional Care and Use of Animals of Nippon Shinyaku Co., Ltd.

Measurement of intracellular cAMP

Chinese hamster ovary (CHO) cells expressing the human EP_3 receptor were produced as previously described (Kuwano et al., 2007), cultured in 24-well plates (1×10^5 cells/well), washed with Hanks' balanced salt solution buffered with 10 mM HEPES, pH 7.4, and preincubated at 37°C for 60 min in the same solution containing the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (500 μM). Then test agent and 1 μM forskolin were added to each well and, after incubation for a further 15 min at 37°C,

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

the reaction was terminated by the addition of perchloric acid solution and the quenched reaction mixture was frozen for 2 h at –80°C. The thawed reaction mixture was centrifuged, a sample of supernatant was removed, and the cAMP in the supernatant was measured with an ELISA kit (GE Healthcare) according to the manufacturer's instructions.

Rat model of monocrotaline-induced pulmonary hypertension

Pulmonary artery hypertrophy and relaxation response, hemodynamic measurements, and assessment of right ventricular hypertrophy

Rats weighing 180–210 g were divided into groups and given a single subcutaneous injection of 40 mg/kg MCT, after which they were orally administered NS-304 at 1 mg/kg, beraprost at 0.1 mg/kg, or vehicle twice daily for 19 days.

Survival

Rats weighing 250–300 g were divided into groups and given a single subcutaneous injection of 60 mg/kg MCT, after which they were orally administered NS-304 at 1 mg/kg or vehicle twice daily for 45 days.

Preparation of pulmonary arteries for measurement of relaxant response to acetylcholine

The day after the final administration of drug to MCT-treated rats, the right main pulmonary arteries (2 mm external diameter) were isolated and cut into rings 3-4 mm in length. The arterial rings were precontracted with 10 μM $PGF_{2\alpha}$ and the relaxant responses induced by various concentrations of acetylcholine were measured as described below and

expressed relative to the papaverine-induced relaxant response.

Assessment of pulmonary arterial wall hypertrophy

Histological analysis of the medial wall thickness of the peripheral pulmonary arteries was performed as described by Ono and Voelkel (1991), with some modifications. In brief, the lungs were immersed in 10% neutral buffered formalin and embedded in paraffin blocks. Four-micrometer sections cut from the middle region of the left lung were stained with elastin van Gieson stain and examined under a microscope at ×400 magnification. For each lung section, 10 muscular arteries showing closed, circular sections 25–100 μm in external diameter were selected at random and their external diameter and medial wall thickness were measured. The percent medial wall thickness was calculated as [(medial wall thickness × 2)/external diameter] × 100.

Hemodynamic measurements and assessment of right ventricular hypertrophy

A polyethylene tube was inserted through the right jugular vein into the right ventricle under urethane anesthesia and connected to a pressure transducer (model TP-300T; Nihon Kohden, Tokyo, Japan). The right ventricular systolic pressure (RVSP) was measured with the pressure transducer connected to a carrier amplifier (model AP-641G; Nihon Kohden). Heart rate (HR) was measured with a tachometer (model AT-601G; Nihon Kohden). After the hemodynamic measurements had been made, the heart was removed and the right ventricular free wall and the left ventricle plus septum were isolated and weighed. The ratio of the weight of the right ventricle to that of the left ventricle plus septum

(RV/(LV+S)) was calculated as an index of right ventricular hypertrophy (RVH).

Measurement of relaxant and contractile responses in pulmonary arteries

Large pulmonary arteries (LPA, main arteries; external diameter ≈2 mm) and small pulmonary arteries (SPA, second or third branches; external diameter ≈0.3 mm) were isolated. The arteries were cut into rings 3-4 mm and 1 mm in length, respectively. LPA without endothelium (LPA(-)) was prepared from LPA with endothelium (LPA(+)) by gently rubbing the intimal surface of LPA(+) with cotton thread and briefly washing the surface with buffer. Because SPA was too small to keep the endothelium intact, only SPA without endothelium (SPA(-)) was prepared. Arterial rings were mounted between two wires in a 37 °C organ bath containing modified Krebs-Ringer bicarbonate solution, pH 7.4. One wire was fixed and the other was connected to an isometric force-displacement transducer (model T7-30-240; Orientec, Tokyo, Japan) and a carrier amplifier (model AP-621G; Nihon Kohden). At the end of each experiment, 100 μM papaverine was applied to produce maximal relaxation. For observation of relaxant responses, arterial rings were precontracted with 10 μM $PGF_{2\alpha}$ before the induction of relaxation by test drugs. The drugs were applied directly to the arterial preparations in cumulative concentrations. Contraction was induced with test drugs. For observation of the effect of an EP_3 receptor antagonist, DBTSA, on the relaxant and contractile responses, arterial rings were treated with the antagonist at a concentration of 3 μM before measurement of the response. For determination of the effect of MCT on contractile and relaxant responses in LPA(+), LPA was isolated from rats 19 or 20 days after injection of 40 mg/kg MCT and the responses

measured as described above.

Statistical analysis

First, the homogeneity of variance between pairs of groups was analyzed by the F-test. When the variance was homogenous, statistical differences between groups were analyzed by Student's *t*-test. When the variance was not homogenous, statistical differences were analyzed by the Welch test. The statistical significance of differences among three or more groups was evaluated by Dunnett's test. Survival curves for both groups were calculated by the Kaplan-Meier method and compared by the log-rank test. A *P* value of less than 0.05 was considered statistically significant. All analyses were performed with SAS version 8.2 (SAS Institute Inc., Cary, NC).

Results

Relaxant response to acetylcholine in the pulmonary artery

Acetylcholine induced vasodilation of the pulmonary artery in a concentration-dependent manner in normal rats (Fig. 1A). This relaxant response was markedly attenuated in MCT-treated rats (MCT group). The maximum relaxant responses in normal and MCT-treated rats were 81% and 25%, respectively, of that induced by papaverine. Repeated treatment of MCT-treated rats with NS-304 significantly suppressed this deterioration of the relaxant response, producing a recovery of the maximum response to approximately 50% of that induced by papaverine.

Hypertrophy of the pulmonary arterial wall

MCT treatment of rats induced marked and significant hypertrophy of the pulmonary arterial wall, the percent wall thickness being 32.2 ± 2.5 in the MCT group versus 16.2 ± 1.0 in the normal group (Fig. 1B), while the percent wall thickness in the NS-304 group, at 23.5 ± 1.5, was significantly lower than in the MCT group. Representative photographs of a pulmonary arterial cross-section for each group also show that the hypertrophy of the arterial wall was markedly reduced by treatment with NS-304 (Fig. 1C).

Right ventricular systolic pressure and right ventricular hypertrophy

RVSP in the MCT group, at 68 ± 4 mmHg, was significantly higher than the value of 36 ± 1 mmHg observed in the normal group (Fig. 2A). Both the NS-304 and the beraprost group demonstrated a significantly lower RVSP than the MCT group, with values of 51 ± 2 and

57 ± 3 mmHg, respectively. The RV/(LV+S) ratio in the MCT group, 0.500 ± 0.027, was also significantly higher than the value of 0.279 ± 0.006 observed in normal group (Fig. 2B). Treatment with NS-304 significantly reduced the ratio to 0.380 ± 0.019, while treatment with beraprost produced no significant decrease (0.441 ± 0.026). There were no significant differences in HR among the groups (Fig. 2C).

Effect of NS-304 on survival

The survival rate of MCT-treated rats at 45 days was 33%, and NS-304 significantly increased this survival rate to 73% (Fig. 2D; *P*=0.0307, log-rank test).

Relaxant response of pulmonary artery preparations to IP receptor agonists

MRE-269 induced concentration-dependent vasodilation in LPA(+), LPA(-) and SPA(-) (Fig. 3A). There were no significant differences in the concentration-response curves for MRE-269 between LPA(-) and SPA(-), but MRE-269-induced vasodilation in LPA was slightly attenuated by endothelium removal at higher concentrations. Although beraprost induced vasodilation in LPA(+), LPA(-), and SPA(-), the relaxant response was attenuated at higher concentrations in all three artery preparations (Fig. 3B). Beraprost-induced vasodilation in LPA was markedly attenuated by endothelium removal, the maximum relaxation at 3 μM being significantly reduced from 81.3 ± 3.5 to 55.3 ± 5.1%. Beraprost-induced vasodilation in SPA(-) was less than in LPA, the maximum relaxation at 3 μM being only 21.5 ± 3.7%. The rank order of the strength of beraprost-induced vasodilation for the three pulmonary artery preparations was LPA(+) > LPA(-) > SPA(-).

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Iloprost also induced concentration-dependent vasodilation in all three artery preparations (Fig. 3C), but endothelium removal had no significant effect on iloprost-induced vasodilation in LPA. The concentration-response curve for iloprost in SPA(-) was shifted to the right compared with the curve for LPA(-). The rank order of the strength of iloprost-induced vasodilation was LPA(+)≈LPA(-) > SPA(-).

EP_3-receptor-agonist-mediated contraction in pulmonary artery preparations

The EP_3 receptor agonist sulprostone induced concentration-dependent vasoconstriction in LPA(+), LPA(-) and SPA(-) (Fig. 4A), and the vasoconstriction in SPA(-) was significantly greater than in LPA(-). The EP_3 receptor antagonist DBTSA attenuated sulprostone-induced vasoconstriction in LPA(-) (Fig. 4B). Beraprost and sulprostone decreased cAMP production evoked by forskolin in CHO cells expressing the human EP_3 receptor (Fig. 5A). This finding indicates that beraprost has EP_3 receptor agonist activity. Iloprost also showed EP_3 receptor agonism (data not shown). Beraprost induced vasoconstriction in LPA(-) (Fig. 5B) and SPA(-) (Fig. 5C), and the EP_3 receptor antagonist DBTSA suppressed the vasoconstriction.

Effect of an EP_3 antagonist on the relaxation of pulmonary artery preparations induced by IP receptor agonists

The EP_3 receptor antagonist DBTSA had no significant effect on the concentration-response curve for MRE-269-induced vasodilation in SPA(-) (Fig. 6A). In contrast, beraprost-induced vasodilation in SPA was enhanced by treatment with DBTSA,

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

and the maximum relaxation induced by beraprost, observed at 3 μM, was significantly increased from 27.4 ± 7.7 to 65.1 ± 6.0% by DBTSA treatment (Fig. 6B). Beraprost-induced vasoconstriction occurred at beraprost concentrations above 3 μM in spite of DBTSA treatment, as evidenced by the observed attenuation of the relaxant response. Although the vasodilation induced by iloprost at lower concentrations was enhanced by DBTSA treatment, iloprost at concentrations greater than 1 μM induced marked vasoconstriction (Fig. 6C).

Effects of MRE-269, beraprost and iloprost on LPA from MCT-treated rats

Vasoconstriction induced by sulprostone, beraprost, or iloprost in rat LPA(+) was increased by treatment of rats with MCT, but MRE-269 induced no vasoconstriction in either normal or MCT-treated rats (Fig. 7). The vasodilation induced by beraprost or iloprost, but not that induced by MRE-269, was more strongly attenuated in LPA from MCT-treated rats than from normal rats (Fig. 8).

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

NS-304 is an orally available and long-acting IP receptor agonist prodrug, and its active form, MRE-269, is a highly selective IP receptor agonist (Kuwano et al., 2007). In the present study we show that, in rats with MCT-induced PH, twice-daily treatment with NS-304 ameliorated the impaired relaxant response to acetylcholine in pulmonary artery preparations and reduced the hypertrophy of the pulmonary arterial wall induced by MCT. These results suggest that NS-304 ameliorates endothelial dysfunction and inhibits the pathological proliferation of intimal smooth muscle cells in the injured arteries. NS-304 also significantly reduced the RVH and RVSP without affecting the HR and significantly increased the survival rate of MCT-treated rats. The positive effect of NS-304 on survival was similar to that observed in this rat PH model after transfer of the PGI_2 synthase gene (Nagaya et al., 2000) or administration of beraprost (Itoh et al., 2004). Our results show that NS-304 suppressed various pathological processes associated with PH in rats.

Although 0.1 mg/kg is likely to be the optimal dose of beraprost for the amelioration of MCT-induced PH in rats (Miyata et al., 1996; Ueno et al., 2002), in the present study, beraprost at this dosage significantly reduced the RVSP but not the RVH. A pharmacokinetic study of NS-304 in humans showed a long plasma elimination half-life for MRE-269 (Kuwano et al., 2007), approximately eight times that of beraprost (Toda N, 1988; Demolis et al., 1993). In contrast, in rats the elimination half-life of beraprost was slightly longer than that of MRE-269 after oral administration of NS-304 (Matsumoto et al., 1998; Kuwano et al., 2007). Even though there is almost no difference between the elimination half-lives of MRE-269 and beraprost in rats, MRE-269 was no less effective

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

than beraprost in MCT-treated rats. Because the binding affinity of MRE-269 for the rat IP receptor (K_i = 220 nM) is about one-tenth that of beraprost (K_i = 19 nM) (Kuwano et al., 2007), the dose of NS-304 used was 10 times the dose of beraprost used. The binding affinity of MRE-269 (K_i = 20 nM) for the human IP receptor is about as high as that of beraprost (K_i = 39 nM) (Kuwano et al., 2007). Differences in the potency of MRE-269 are probably due to species differences in the binding affinity of MRE-269 for the IP receptor. Similar species differences have been reported for a non-prostanoid IP receptor agonist, octimibate (Merritt et al., 1991).

We have previously found that the efficacy of beraprost in increasing femoral skin blood flow in rats is lower than that of NS-304, and we suggested that the EP_3 receptor agonist activity of beraprost attenuates its efficacy at the IP receptor (Kuwano et al., 2007). To investigate the effect on vasodilation of differences among the receptor selectivity profiles of MRE-269, beraprost, and iloprost, we assessed the relaxant response to these compounds in pulmonary artery preparations. Although the receptor selectivity profiles of MRE-269 and PGI_2 analogs among rat prostanoid receptors are unknown, we have previously shown that MRE-269 is highly selective for the human IP receptor (K_i for IP= 20 nM; K_i for EP_3 = >10 μM), while beraprost (K_i for IP= 39 nM; K_i for EP_3 = 680 nM) and iloprost (K_i for IP= 11 nM; K_i for EP_1 = 11 nM; K_i for EP_3 = 56 nM) show poor selectivity for the human IP receptor because of their relatively high affinities for human EP subtypes (Abramovitz et al., 2000; Kuwano et al., 2007). In the present study, the relaxant responses of the pulmonary artery preparations to the compounds were clearly different. MRE-269 induced vasodilation equally in LPA(-) and SPA(-), whereas beraprost and iloprost induced

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

less vasodilation in SPA(-) than in LPA(-). The efficacy of MRE-269 in inducing vasodilation in pulmonary arteries was greater than that of beraprost, especially in SPA(-).

EP_3 receptor agonists cause pulmonary arterial contraction (Qian et al., 1994; Norel et al., 2004), and EP_3-receptor-mediated agonism interferes with IP-receptor-mediated vasorelaxation (Jones et al., 1997; Chan et al., 2004). In the present study, an EP_3 receptor agonist, sulprostone, induced vasoconstriction in both LPA(-) and SPA(-), and the vasoconstriction observed in SPA(-) was more potent than that observed in LPA(-). Sulprostone-induced vasoconstriction in LPA(-) was suppressed by an EP_3 receptor antagonist, DBTSA, which has a high affinity for the human EP_3 receptor (K_i=25 nM) (Gallant et al., 2002). These results suggest that EP_3-receptor-mediated vasoconstriction in SPA is greater than in LPA, and therefore that the EP_3 receptor density in SPA is likely to be higher than in LPA. In the present study, beraprost and iloprost showed apparent EP_3 receptor agonism, as judged by their suppression of cAMP production in cells expressing the EP_3 receptor. Beraprost induced vasoconstriction in both LPA(-) and SPA(-), and DBTSA completely inhibited this vasoconstriction. Moreover, treatment with DBTSA enhanced the vasodilation induced by beraprost or iloprost in SPA, but not the vasodilation induced by MRE-269. These findings indicate that the vasodilation in SPA induced by beraprost or iloprost, but not that induced by MRE-269, is counteracted by EP_3 receptor agonism. Beraprost induced vasoconstriction in LPA and SPA at concentrations of more than 10 μM; in contrast, significant attenuation of beraprost-induced vasodilation started at concentrations as low as 1 nM. In the contractile response induced by beraprost, because IP-receptor-mediated vasodilation is expected to

occur concomitantly with the vasoconstriction, it was likely that vasodilation canceled out the vasoconstriction at lower concentrations of beraprost. Consistent with this, beraprost-induced vasoconstriction was observed only at higher concentrations. Because the progression of PAH mainly affects the small pulmonary arteries, MRE-269, which can equally relax both LPA and SPA, has an advantage over beraprost and iloprost, which preferentially relax LPA, as a medication for PAH.

Iloprost induced equal vasodilation in LPA irrespective of the presence or absence of endothelium, and MRE-269-induced vasodilation in LPA was slightly attenuated by endothelium removal only at higher concentrations. However, there was no significant difference in the maximum relaxation induced by either MRE-269 or iloprost in the presence and in the absence of endothelium. In contrast, the vasodilation induced by beraprost was markedly attenuated by endothelium removal. Because PAH is usually accompanied by endothelial dysfunction, the ability to induce an endothelium-independent relaxant response is a desirable pharmacological characteristic in a drug intended for the treatment of PAH. Treatment of rats with MCT led to endothelial dysfunction in the pulmonary arteries, as well as to an increase in EP_3-receptor-mediated vasoconstriction. MRE-269 induced vasodilation equally in LPA from normal and MCT-treated rats. In contrast, vasodilation induced by beraprost or iloprost was markedly attenuated in LPA from MCT-treated rats compared with normal rats. Vasoconstriction induced by beraprost or iloprost was intensified by treatment with MCT. This vasoconstriction seems to contribute to the attenuation of vasodilation induced by these compounds. Furthermore, repeated treatment with NS-304 ameliorated endothelial dysfunction in the pulmonary

arteries of MCT-treated rats. We have therefore shown that NS-304 could both protect the endothelium and relax pulmonary arteries with impaired endothelium. Although the PGI_2 analogs iloprost and beraprost have similar chemical structures, unexpectedly they showed different dependencies of their vasodilation activity on the presence or absence of endothelium. The reason for this is unknown, but it may be related to the fact that iloprost has agonist activity at the EP_1 receptor in addition to the IP and EP_3 receptors (Abramovitz et al., 2000). In the treatment of PAH with either PGI_2 receptor agonist, dose escalation is commonly required (Barst et al., 1996), and this is likely to bring about significant EP_3 agonism, thereby attenuating IP-receptor-mediated vasorelaxation.

In conclusion, NS-304 was found to ameliorate various pathological processes associated with PH in MCT-treated rats, and its active form, MRE-269, showed suitable pharmacological characteristics in the vasodilation of pulmonary artery preparations for a drug intended to treat PAH. Its favorable pharmacological profile is based in part on its high selectivity for the IP receptor. NS-304 is therefore a promising alternative medication for PAH with prospects for good patient compliance.

Acknowledgments

We thank Dr. Gerald E. Smyth for helpful suggestions during the preparation of the manuscript.

References

Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, Belley M, Gallant M, Dufresne C, Gareau Y, Ruel R, Juteau H, Labelle M, Ouimet N, and Metters KM (2000) The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. *Biochim Biophys Acta* **1483:**285-293.

Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH, Koerner SK, Langleben D, Keller CA, Murali S, Uretsky BF, Clayton LM, Jöbsis MM, Blackburn SD, Shortino D, and Crow JW (1996) A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl J Med* **334:**296-301.

Chan KM and Jones RL (2004) Partial agonism of taprostene at prostanoid IP receptors in vascular preparations from guinea-pig, rat, and mouse. *J Cardiovasc Pharmacol* **43:**795-807.

Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM, and Loyd JE (1992) An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med* **327:**70-75.

Demolis JL, Robert A, Mouren M, Funck-Brentano C, and Jaillon P (1993)

Pharmacokinetics and platelet antiaggregating effects of beraprost, an oral stable prostacyclin analogue, in healthy volunteers. *J Cardiovasc Pharmacol* **22:**711-716.

Farber HW and Loscalzo J (2004) Mechanisms of disease: pulmonary arterial hypertension. *N Engl J Med* **351:**1655-1665.

Galiè N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, and Simonneau G; Sildenafil Use in Pulmonary Arterial Hypertension (SUPER) Study Group (2005) Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* **353:**2148-2157.

Gallant M, Carrière MC, Chateauneuf A, Denis D, Gareau Y, Godbout C, Greig G, Juteau H, Lachance N, Lacombe P, Lamontagne S, Metters KM, Rochette C, Ruel R, Slipetz D, Sawyer N, Tremblay N, and Labelle M (2002) Structure–Activity Relationship of Biaryl Acylsulfonamide Analogues on the Human EP_3 Prostanoid Receptor. *Bioorg Med Chem Lett* **12:**2583–2586.

Hoshikawa Y, Voelkel NF, Gesell TL, Moore MD, Morris KG, Alger LA, Narumiya S, and Geraci MW (2001) Prostacyclin receptor-dependent modulation of pulmonary vascular remodeling. *Am J Respir Crit Care Med* **164:**314-318.

Humbert M, Sanchez O, Fartoukh M, Jagot JL, Le Gall C, Sitbon O, Parent F, and

Simonneau G (1999) Short-term and long-term epoprostenol (prostacyclin) therapy in pulmonary hypertension secondary to connective tissue diseases: results of a pilot study. *Eur Respir J* **13:**1351-1356.

Itoh T, Nagaya N, Fujii T, Iwase T, Nakanishi N, Hamada K, Kangawa K and Kimura H (2004) A combination of oral sildenafil and beraprost ameliorates pulmonary hypertension in rats. *Am J Respir Crit Care Med* **169:** 34-38.

Jones RL, Qian YM, Chan KM, and Yim AP (1997) Relaxant Actions of Nonprostanoid Prostacyclin Mimetics on Human Pulmonary Artery. *J Cardiovasc Pharmacol* **29:**525-535.

Jones RL, Qian YM, Chan KM, and Yim AP (1998) Characterization of a prostanoid EP_3-receptor in guinea-pig aorta: partial agonist action of the non-prostanoid ONO-AP-324. *Br J Pharmacol* **125:**1288-1296.

Kiriyama K, Ushikubi F, Kobayashi T, Hirata M, Sugimoto S, and Narumiya S (1997) Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. *Br J Pharmacol* **122:**217-224.

Kuwano K, Hashino A, Asaki T, Hamamoto T, Yamada T, Okubo K, and Kuwabara K (2007)

2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamid e (NS-304), an Orally Available and Long-Acting Prostacyclin Receptor Agonist Prodrug. *J Pharmacol Exp Ther* **322:**1181-1188.

Mathai SC, Girgis RE, Fisher MR, Champion HC, Housten-Harris T, Zaiman A, and Hassoun PM (2007) Addition of sildenafil to bosentan monotherapy in pulmonary arterial hypertension. *Eur Respir J* **29:**469-475.

Matsumoto K, Tajima A, Hirano Y, and Ohno K (1989) Pharmacokinetics and biotransformation of beraprost sodium I: Plasma level profile of beraprost sodium in rat. *Drug Metab Pharmacokinet* **4:** 713-725.

McLaughlin VV, Oudiz RJ, Frost A, Tapson VF, Murali S, Channick RN, Badesch DB, Barst RJ, Hsu HH, and Rubin LJ (2006) Randomized study of adding inhaled iloprost to existing bosentan in pulmonary arterial hypertension. *Am J Respir Crit Care Med* **174:** 1257-1263.

Merritt JE, Hallam TJ, Brown AM, Boyfield I, Cooper DG, Hickey DM, Jaxa-Chamiec AA, Kaumann AJ, Keen M, Kelly E, Kozlowski U, Lynham JA, Moores KE, Murray KJ, MacDermot J, and Rink TJ (1991) Octimibate, a potent non-prostanoid inhibitor of platelet aggregation, acts via the prostacyclin receptor. *Br J Pharmacol* **102:**251-259.

Miyata M, Ueno Y, Sekine H, Ito O, Sakuma F, Koike H, Nishio S, Nishimaki T, Kasukawa R (1996) Protective effect of beraprost sodium, a stable prostacyclin analogue, in development of monocrotaline-induced pulmonary hypertension. *J Cardiovasc Pharmacol* **27:** 20-26.

Nagaya N, Yokoyama C, Kyotani S, Shimonishi M, Morishita R, Uematsu M, Nishikimi T, Nakanishi N, Ogihara T, Yamagishi M, Miyatake K, Kaneda Y, and Tanabe T (2000) Gene transfer of human prostacyclin synthase ameliorates monocrotaline-induced pulmonary hypertension in rats. *Circulation* **102:**2005-2010.

Namba T, Oida H, Sugimoto Y, Kakizuka A, Negishi M, Ichikawa A, and Narumiya S (1994) cDNA cloning of a mouse prostacyclin receptor. Multiple signaling pathways and expression in thymic medulla. *J Biol Chem* **269:**9986-9992.

Narumiya S, Sugimoto Y, and Ushikubi F (1999) Prostanoid receptors: structures, properties, and functions. *Physiol Rev* **79:**1193-1226.

Norel X, de Montpreville V, and Brink C (2004) Vasoconstriction induced by activation of EP_1 and EP_3 receptors in human lung: effects of ONO-AE-248, ONO-DI-004, ONO-8711 or ONO-8713. *Prostaglandins Other Lipid Mediat* **74:**101-112.

Olschewski H, Simonneau G, Galiè N, Higenbottam T, Naeije R, Rubin LJ, Nikkho S,

Speich R, Hoeper MM, Behr J, Winkler J, Sitbon O, Popov W, Ghofrani HA, Manes A, Kiely DG, Ewert R, Meyer A, Corris PA, Delcroix M, Gomez-Sanchez M, Siedentop H, and Seeger W (2002) Inhaled iloprost for severe pulmonary hypertension. *N Engl J Med* **347:**322-329.

Ono S and Voelkel NF (1991) PAF antagonists inhibit monocrotaline-induced lung injury and pulmonary hypertension. *J Appl Physiol* **71:**2483-2492.

Qian YM, Jones RL, Chan KM, Stock AI, and Ho JK (1994) Potent contractile actions of prostanoid EP_3-receptor agonists on human isolated pulmonary artery. *Br J Pharmacol* **113:**369-374.

Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK, Levy PC, Reid LM, Vreim CE and Williams GW (1987) Primary pulmonary hypertension. A national prospective study. *Ann Intern Med* **107:**216-223.

Rubin LJ (1997) Primary pulmonary hypertension. *N Engl J Med* **336:**111-117.

Rubin LJ, Badesch DB, Barst RJ, Galiè N, Black CM, Keogh A, Pulido T, Frost A, Roux S, Leconte I, Landzberg M, and Simonneau G (2002) Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med* **346:**896-903.

Runo JR and Loyd JE (2003) Primary pulmonary hypertension. *Lancet* **361:**1533-1544.

Simonneau G, Barst RJ, Galie N, Naeije R, Rich S, Bourge RC, Keogh A, Oudiz R, Frost A, Blackburn SD, Crow JW, and Rubin LJ (2002) Continuous subcutaneous infusion of treprostinil, a prostacyclin analogue, in patients with pulmonary arterial hypertension: a double-blind, randomized, placebo-controlled trial. *Am J Respir Crit Care Med* **165:**800-804.

Sitbon O, Humbert M, Nunes H, Parent F, Garcia G, Hervé P, Rainisio M, and Simonneau G. (2002) Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival. *J Am Coll Cardiol* **40:**780-788.

Toda N (1988) Beraprost sodium. *Cardiovasc Drug Rev* **6:**222-238.

Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, and Voelkel NF (1999) Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. *Am J Respir Crit Care Med* **159:**1925-1932.

Ueno M, Miyauchi T, Sakai S, Yamauchi-Kohno R, Goto K, and Yamaguchi I (2002) A combination of oral endothelin-A receptor antagonist and oral prostacyclin analogue is superior to each drug alone in ameliorating pulmonary hypertension in rats. *J Am Coll*

Cardiol **40:**175-181.

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig. 1. Effects of NS-304 on (A) acetylcholine-induced vasodilation of pulmonary arteries and (B, C) hypertrophy of the pulmonary arterial wall in monocrotaline (MCT)-treated rats. NS-304 was orally administered to MCT -treated rats at 1 mg/kg twice daily for 19 days. (A) Right main pulmonary arterial rings were precontracted with 10 μM $PGF_{2\alpha}$ and the relaxant responses induced by various concentrations of acetylcholine were measured and expressed as a percentage of that induced by 100 μM papaverine (MCT and NS-304, n=6; Normal, n=7). (B) (MCT and NS-304, n=10; Normal, n=8). (C) Representative photomicrographs of cross-sections of peripheral pulmonary arteries from each group. Bar=25 μm. Data are shown as the mean±SEM. ##, $P<0.01$ vs. Normal by Student's *t*-test or the Welch test; *, $P<0.05$; **, $P<0.01$ vs. MCT by Student's *t*-test or the Welch test.

Fig. 2. Effects of NS-304 and beraprost on (A) right ventricular systolic pressure, (B) right ventricular hypertrophy (assessed as RV/(LV+S), the ratio of the weight of the right ventricle to that of the left ventricle plus the septum) and (C) heart rate in MCT-treated rats. NS-304 at 1 mg/kg or beraprost at 0.1 mg/kg was orally administered to MCT-treated rats twice daily for 19 days. Data are shown as the mean±SEM. ##, $P<0.01$ vs. Normal by the Welch test; *, $P<0.05$; **, $P<0.01$ vs. MCT by Dunnett's test; NS, not significant. Statistical differences in RVH and RVSP between Normal and MCT groups were evaluated by the Welch test, statistical differences in RVH and RVSP among the MCT, NS-304 and beraprost groups were evaluated by Dunnett's test, and statistical difference in the HR among four groups (three groups vs. Normal) were evaluated by Dunnett's test

(n=12). (D) Effect of NS-304 on the survival of MCT-treated rats. NS-304 administration was started on day 0. Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test (n=15).

Fig. 3. Effect of MRE-269 (A), beraprost (B) and iloprost (C) on relaxation in rat large pulmonary arteries with endothelium (LPA(+)) and without endothelium (LPA(-)) and small pulmonary arteries without endothelium (SPA(-)) precontracted with 10 μM $PGF_{2\alpha}$. Relaxation is expressed as a percentage of that induced by 100 μM papaverine. Data are shown as the mean±SEM (n=8). *, $P<0.05$; **, $P<0.01$ vs. LPA(-) by Dunnett's test.

Fig. 4. (A) Concentration-response curves for sulprostone-induced contraction in rat pulmonary arteries. Data are shown as the mean±SEM (LPA(+) and SPA(-), n=6; LPA(-), n=5). *, $P<0.05$; **, $P<0.01$ vs. LPA(-) by Dunnett's test. (B) Effect of an EP_3 antagonist, DBTSA, at 3 μM on sulprostone-induced vasoconstriction in SPA(-). Data are shown as the mean±SEM (Control, n=5; EP_3 antagonist, n=6). *, $P<0.05$; **, $P<0.01$ vs. Control by Student's *t*-test.

Fig. 5. (A) Effect of beraprost on cAMP production evoked by forskolin in CHO cells expressing the human EP_3 receptor. Effect of an EP_3 antagonist, DBTSA, on beraprost-induced vasoconstriction in rat LPA(-) (B) and SPA(-) (C). Data are shown as the mean±SEM (LPA(-), n=5; SPA(-), n=6). *, $P<0.05$; **, $P<0.01$ vs. Control by Student's *t*-test or the Welch test.

JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

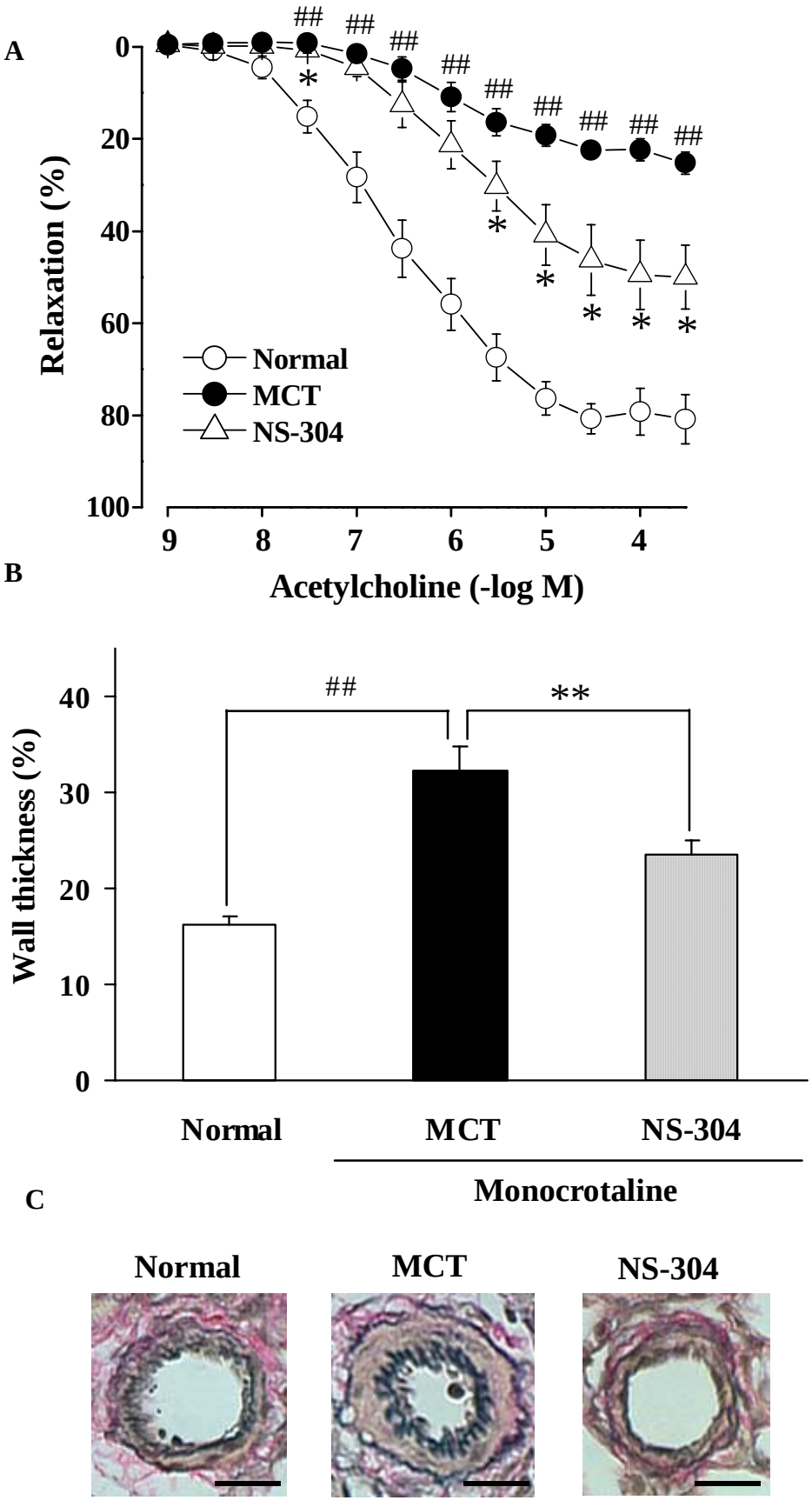
Fig. 6. Effect of an EP_3 antagonist, DBTSA, at 3 μM on relaxation induced by MRE-269 (A), beraprost (B), and iloprost (C) in rat SPA(-) precontracted with 10 μM $PGF_{2\alpha}$. Arterial relaxation is expressed as a percentage of that induced by 100 μM papaverine. Data are shown as the mean±SEM (n=6-8). *, $P<0.05$; **, $P<0.01$ vs. Control by Student's *t*-test or the Welch test.

Fig. 7. Concentration-response curves for contraction induced by sulprostone (A), MRE-269 (B), beraprost (C) and iloprost (D) in LPA(+) from normal and MCT-treated rats. Data are shown as the mean±SEM (n=5 or 6). *, $P<0.05$; **, $P<0.01$ vs. Normal by Student's *t*-test or the Welch test.

Fig. 8. Concentration-response curves for relaxation in rat LPA(+) induced by MRE-269 (A), beraprost (B), and iloprost (C) after precontraction with 10 μM $PGF_{2\alpha}$. LPA(+) was isolated from normal and MCT-treated rats. Data are shown as the mean±SEM (n=8). *, $P<0.05$; **, $P<0.01$ vs. Normal by Student's *t*-test or the Welch test.

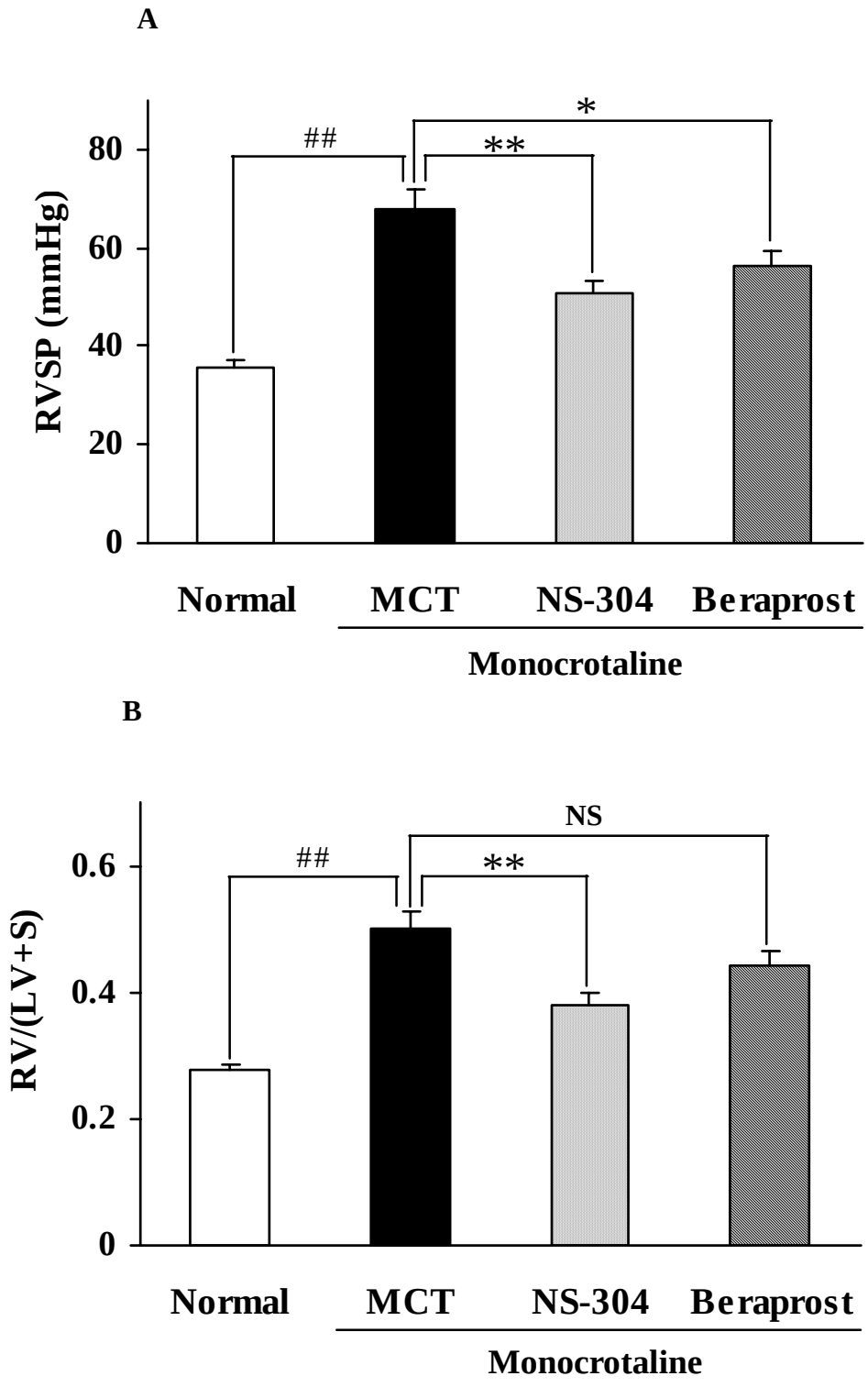
JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 1.



JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

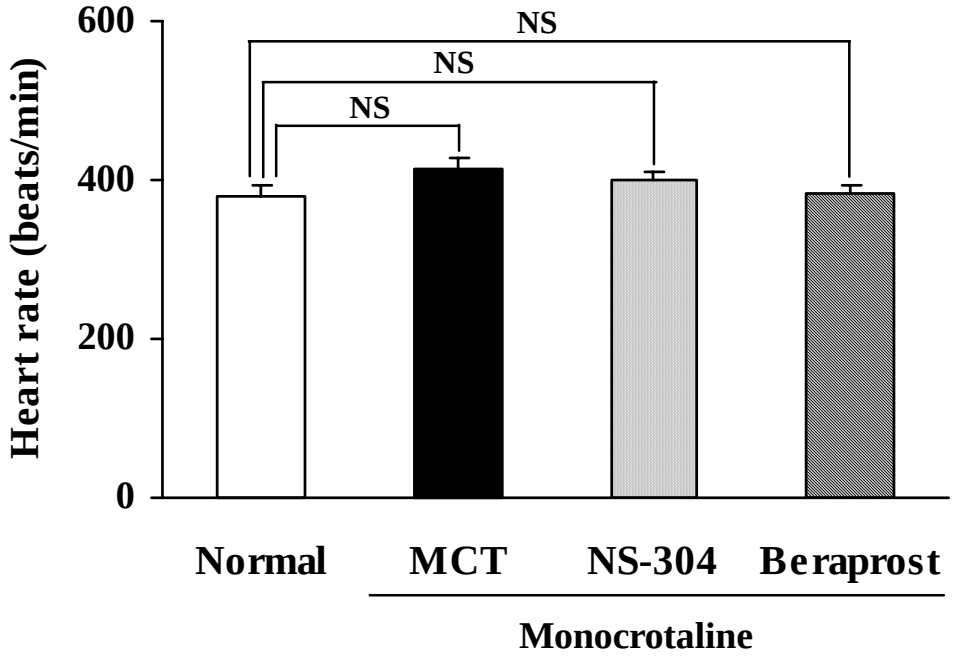
Fig. 2.



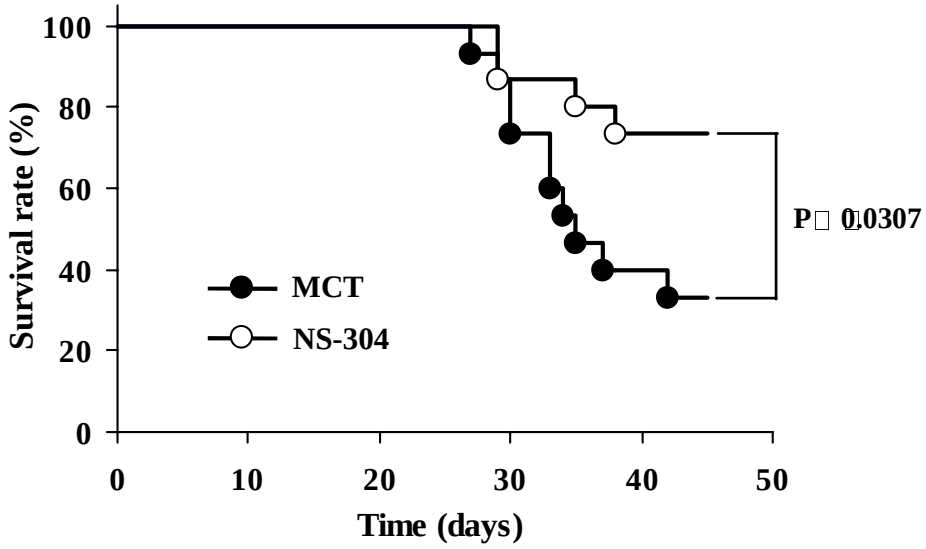
JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 2.

C

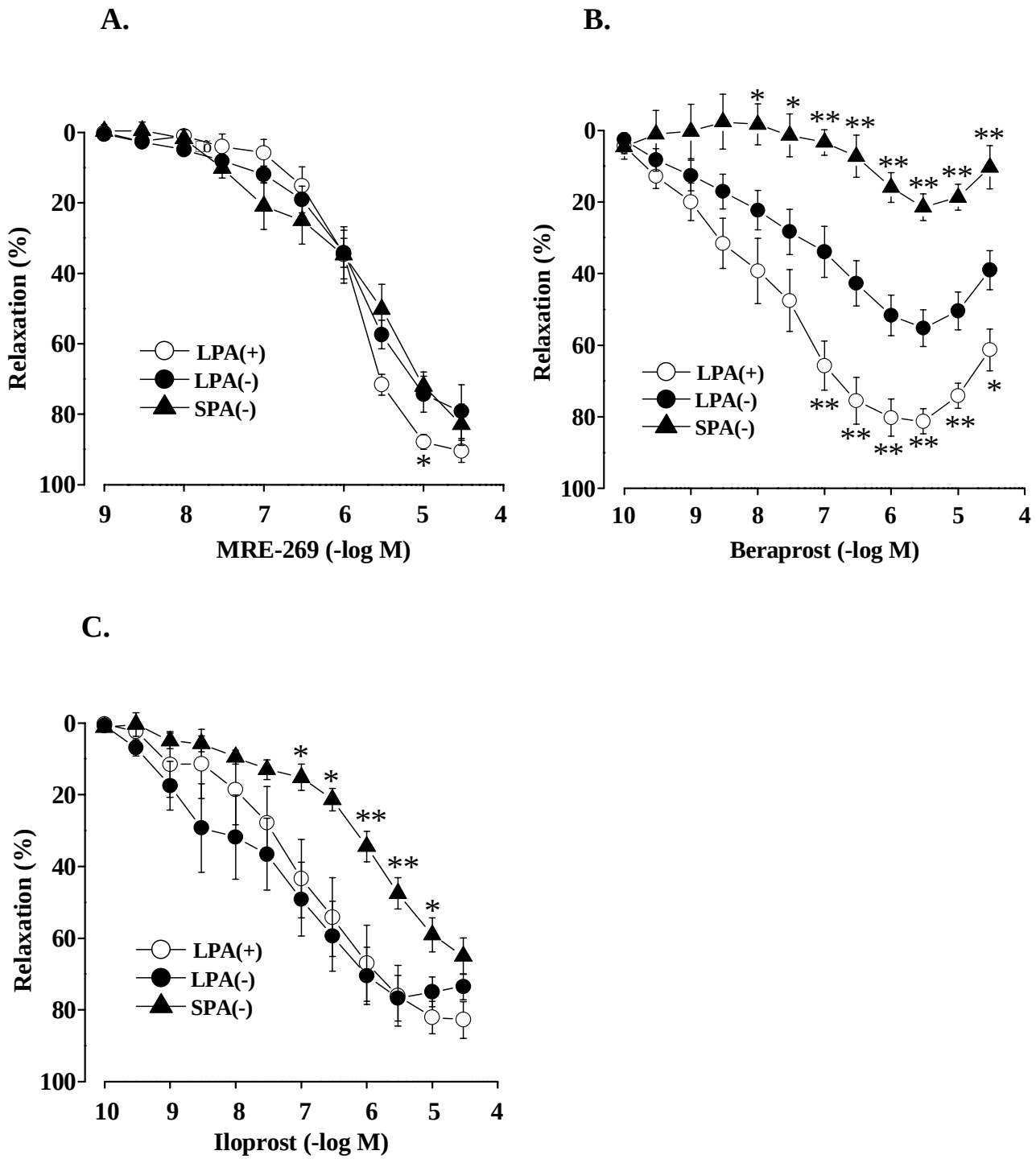


D



JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

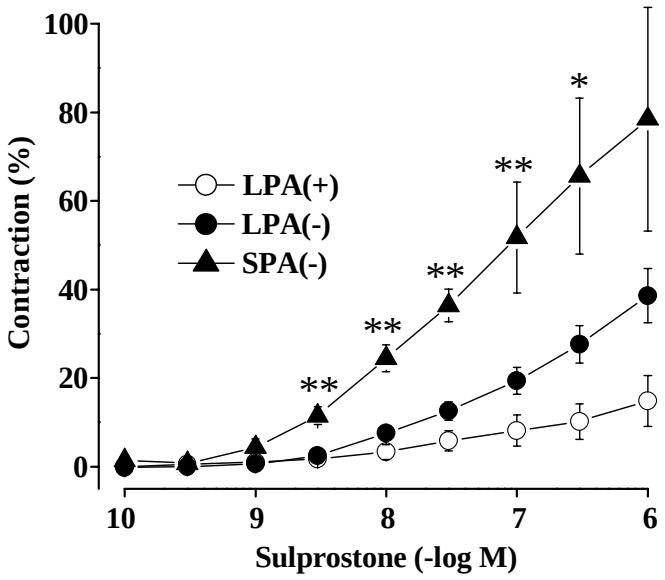
Fig. 3.



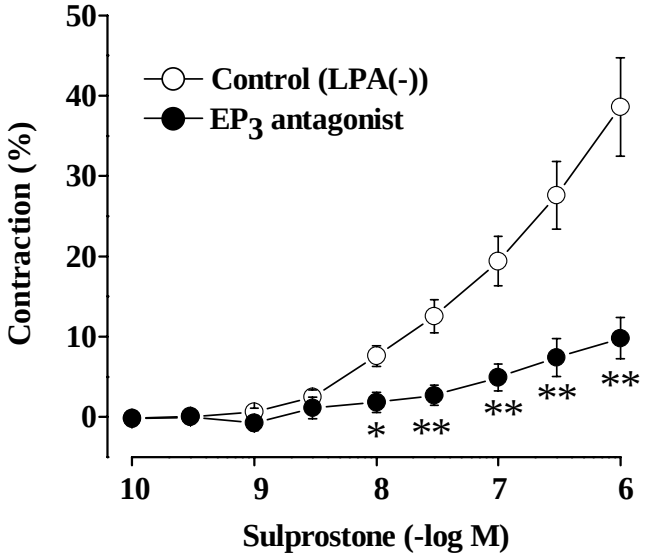
JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4.

A

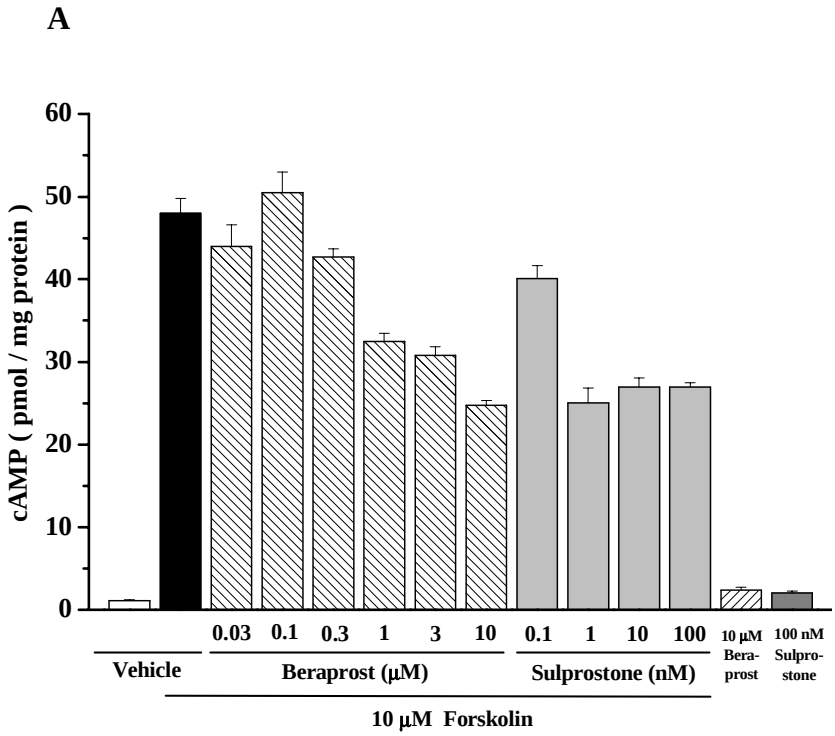


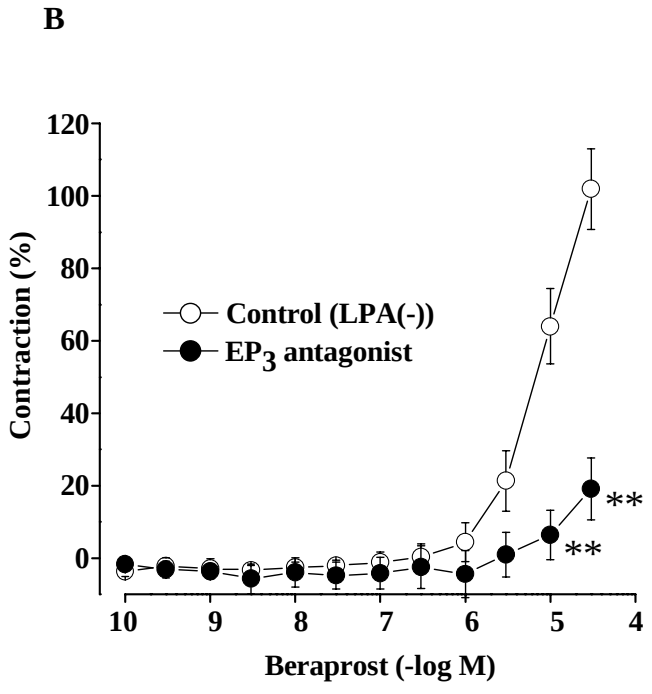
B

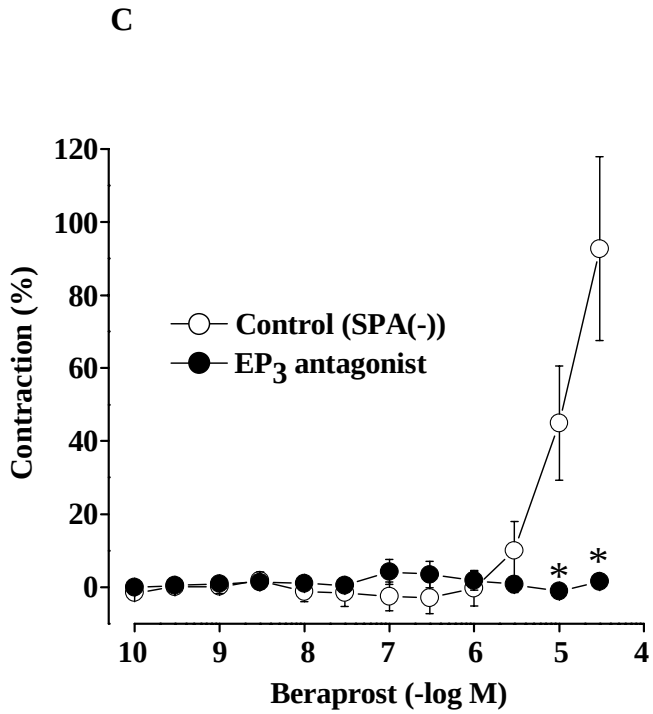


JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 5.

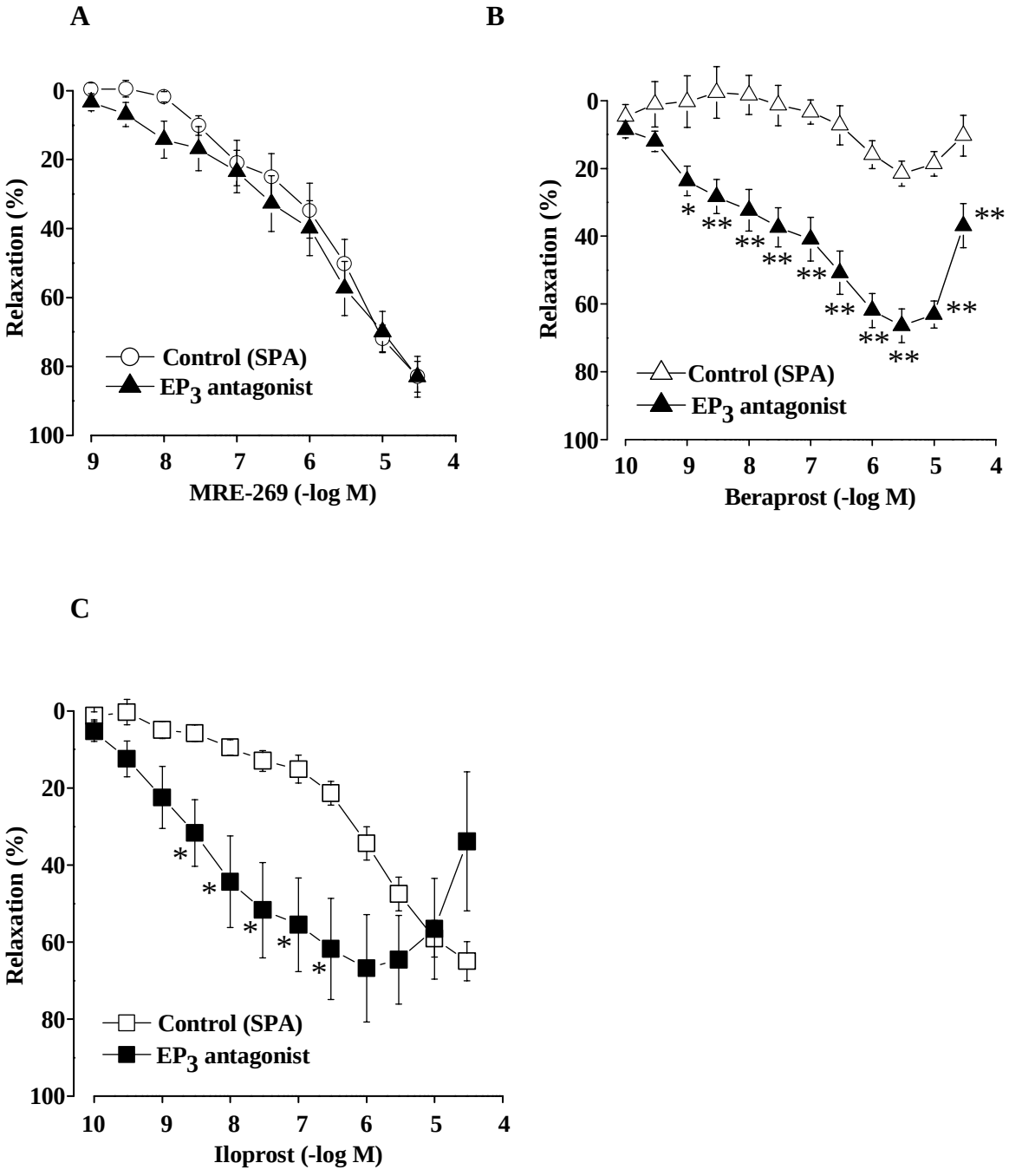






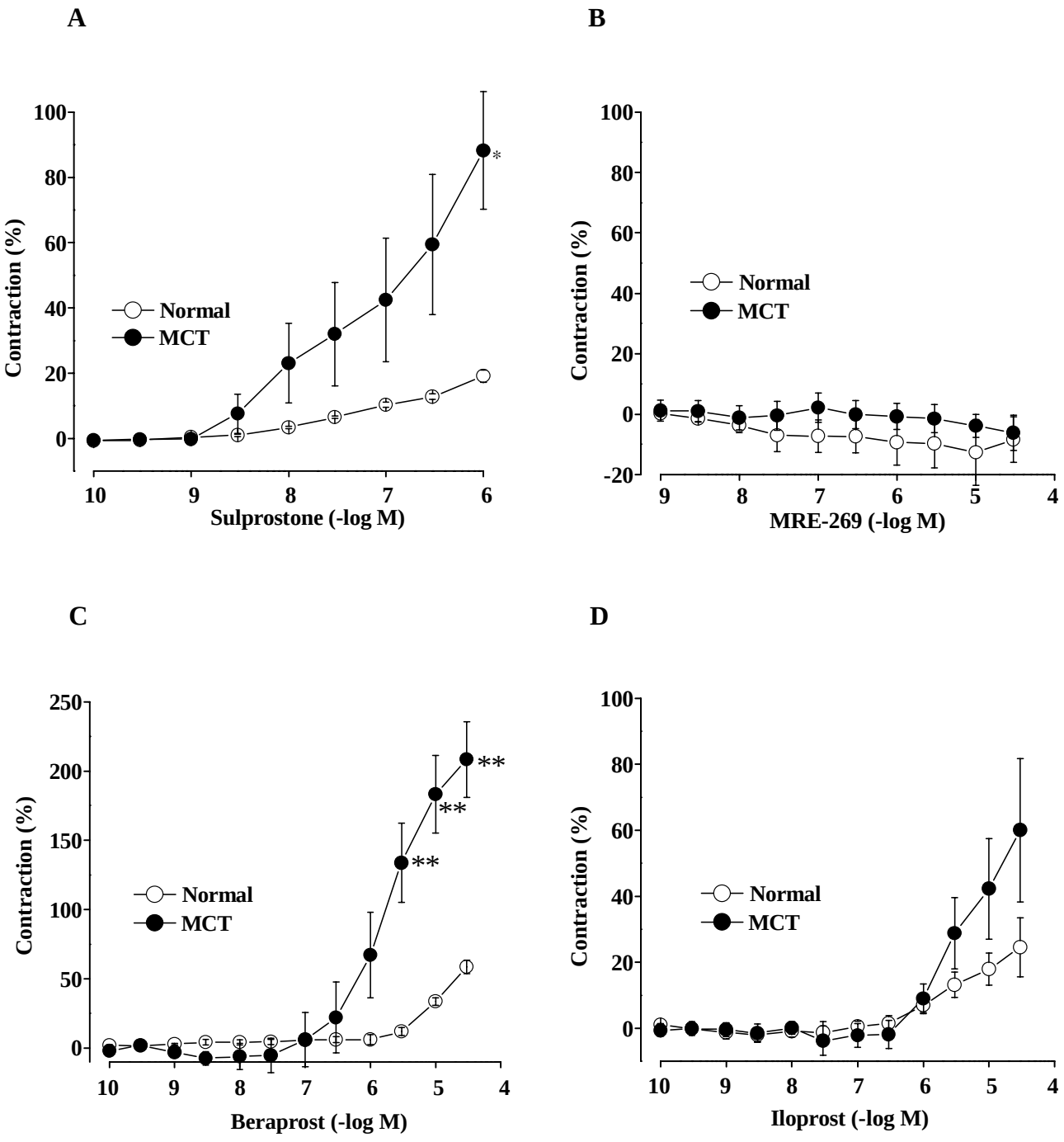
JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 6.



JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 7.



JPET Fast Forward. Published on June 13, 2008 as DOI: 10.1124/jpet.108.138305
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 8.