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In vivo Characterization of the Novel Imidazopyridine BYK191023, a Potent and Highly Selective Inhibitor of Inducible Nitric Oxide Synthase

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Abbreviations:

NOS, nitric oxide synthase; i-, e-, nNOS, inducible, endothelial, neuronal nitric oxide synthase; BYK191023, 2-[2-(4-methoxy-pyridin-2-yl)-ethyl]-3H-imidazo[4,5-b]pyridine; NO_x, nitrate/nitrite; L-NMMA, N^G-monomethyl-L-arginine; MAP, mean arterial pressure

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Abstract

Excessive release of nitric oxide from inducible nitric oxide synthase (iNOS) has been postulated to contribute to pathology in a number of inflammatory diseases. We recently identified imidazopyridine derivatives as a novel class of potent nitric oxide synthase inhibitors with high selectivity for the inducible isoform. In the present study we tested the *in vivo* potency of BYK191023 (2-[2-(4-methoxy-pyridin-2-yl)-ethyl]-3H-imidazo[4,5-b]pyridine), a selected member of this inhibitor class, in three different rat models of lipopolysaccharide-induced systemic inflammation. Delayed administration of BYK191023 dose-dependently suppressed the lipopolysaccharide-induced increase in plasma nitrate/nitrite (NOx) levels with an ED_{50} of 14.9 µmol/kg/h. In a model of systemic hypotension following high dose lipopolysaccharide challenge, curative administration of BYK191023 at a dose that inhibited 83% of the NOx increase completely prevented the gradual decrease in mean arterial blood pressure (MAP) observed in vehicle treated control animals. The vasopressor effect was specific for endotoxaemic animals, since BYK191023 did not affect blood pressure in saline challenged controls. In addition, in a model of lipopolysaccharide-induced vascular hyporesponsiveness, BYK191023 infusion partially restored normal blood pressure responses to norepinephrine and sodium nitroprusside via an L-arginine competitive mechanism. Taken together, BYK191023 is a member of a novel class of highly isoform selective iNOS inhibitors with promising in vivo activity suitable for mechanistic studies on the role of selective iNOS inhibition as well as clinical development.

Introduction

Nitric oxide synthases (NOS) catalyze the conversion of L-arginine to citrulline and nitric oxide. The constitutively expressed endothelial (eNOS) and neuronal (nNOS) NOS isoforms are involved in regulation of vascular tone and neurotransmission, respectively, and release nanomolar concentrations of nitric oxide upon activation by elevated intracellular calcium concentrations (Alderton et al., 2001). In contrast, the inducible NOS isoform (iNOS) is transcriptionally regulated and, once induced by pro-inflammatory stimuli, generates micromolar range quantities of nitric oxide (Stuehr et al., 1991). Although iNOS induction plays a beneficial role in host defence against invading pathogens, uncontrolled release of excessive amounts of nitric oxide from iNOS is postulated to contribute to pathophysiology in a number of acute and chronic inflammatory and autoimmune diseases (Cuzzocrea et al., 2002; Razavi et al., 2004).

Hyperdynamic septic shock is characterized by pathological vasodilatation and reduction of vascular resistance, eventually leading to an uncompensated fall in blood pressure and inadequate organ perfusion. Infusion of catecholamine vasoconstrictors is usually employed to stabilize blood pressure in hyperdynamic septic shock states refractory to volume administration (Beale et al., 2004). Hyporesponsiveness of the large resistance vessels to vasopressor administration is observed in a number of septic patients with hypotension despite sufficient volume substitution (Groeneveld et al., 1986). Consequently, with the aim to maintain adequate systemic blood pressure, either high dose infusion of vasoconstrictors at the cost of excessive peripheral vasoconstriction and increased oxygen debt, or experimental rescue drugs such as vasopressin or methylene blue have to be administered (Stanchina and Levy, 2004; Tsuneyoshi et al., 2001).

It has been postulated that excessive release of nitric oxide from iNOS is causally implicated in detrimental vasodilatation and the loss of vascular responsiveness to vasopressor therapy in septic shock (Tsuneyoshi et al., 1996). Animal models of systemic inflammation mimic certain aspects of human septic shock such as volume resistant hypotension and vascular hyporesponsiveness to vasopressor agents.

Several authors demonstrated the efficacy of different NOS inhibitors to restore normal blood pressure in animal models of lipopolysaccharide-induced hypotension (Ichinose et al., 2003; Rosselet et al., 1998; Ruetten et al., 1996). In addition, inhibition of nitric oxide release proved effective to counteract vascular hyporesponsiveness and vasopressor resistance associated with systemic inflammation in vivo (Cai et al., 1996; Gray et al., 1991) or in isolated vessels in vitro (Hollenberg et al., 1993; Tsuneyoshi, et al., 1996), supporting the concept that inhibition of iNOS could represent an interesting therapeutic target in septic shock. However, the overall effect of iNOS inhibition on morbidity and mortality of septic animals remains controversial, depending on the animal model and the selectivity profile of the employed inhibitor. Whereas inhibitors with a preferential inhibition of the inducible NOS isoform yielded beneficial effects on organ dysfunction or survival (Liaudet et al., 1998; Rosselet et al., 1998; Schwartz et al., 2001; Wu et al., 1995), administration of non-selective NOS inhibitors was associated with unfavourable outcome in several studies of endotoxic shock (Cohen et al., 2000; Liaudet et al., 1998; Schwartz et al., 2001). The hypothesis that non-selective NOS inhibition bears the risk of enhancing inflammation-induced organ dysfunction was confirmed by the unfavourable outcome of a clinical phase III trial on the efficacy of N^G-monomethyl-L-arginine (L-NMMA) in septic shock. Although the actual mechanism underlying the overall adverse effect of L-NMMA remains unknown (Lopez et al., 2004), excessive vasoconstriction of pulmonary or microcirculatory vessels secondary to co-inhibition of physiological nitric oxide release from eNOS could have exerted deleterious effects on organ oxygen supply and cardiovascular function in L-NMMA treated patients (Dellinger and Parrillo, 2004). Therefore, high iNOS selectivity and lack of interference with physiological nitric oxide release from e/nNOS is considered highly desirable for any future drug targeting nitric oxide production in septic patients (Cobb, 2001; Dellinger and Parrillo, 2004).

We recently identified imidazopyridine derivatives as a novel class of iNOS inhibitors with potent enzymatic and cellular activity. BYK191023 (2-[2-(4-methoxy-pyridin-2-yl)-ethyl]-3H-imidazo[4,5-b]pyridine), a member of this novel iNOS inhibitor class, displays L-arginine-competitive inhibition of NOS activity with pIC_{50} values of 7.07 (iNOS), 4.79 (nNOS) and 3.81 (eNOS), respectively, thus reflecting excellent inducible isoform selectivity (Strub et al., 2005). In the present study we assessed the *in vivo* inhibitory potency of BYK191023 on iNOS

activity in lipopolysaccharide-challenged rats. In addition, we tested the therapeutic effect of selective iNOS inhibition by BYK191023 on lipopolysaccharide-induced vascular hyporesponsiveness and systemic hypotension, two aspects of septic shock frequently ascribed to excessive nitric oxide release from iNOS. Our results underpin the value of BYK191023 as a selective pharmacological tool and as a potential novel therapeutic agent in septic shock.

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 220-250 g, were used throughout the experiments. Animals were purchased from Charles River (Sulzfeld, Germany) and kept in our in-house animal facility at a 12 hrs day/night rhythm with free access to standard chow and tap water for at least 4 days prior to inclusion in experiments. All animals received humane care in accordance with the National Institutes of Health guidelines. All experiments were approved by the local ethics committee according to the legal requirements in Germany.

Inhibition of iNOS Activity in Lipopolysaccharide Challenged Rats as Assessed by Determination of Plasma NOx Levels

For all experiments, BYK191023 was dissolved in NaCl 0.9% with 2.5% of polyethylenglycol 400 under acidification to pH 2-3 with 1 N HCl. Lower concentrations were prepared from the stock solution (50 mM) by dilution in NaCl 0.9%.

Rats received an i.v. injection of lipopolysaccharide 0.5 mg/kg in 1 ml/kg, dissolved in NaCl 0.9% + 0.1% hydroxylamine via the tail vein. After 30 min, the animals were anesthetized by i.p. injection of thiopental (Trapanal®, 56 mg/kg), followed by i.m. injection of ketamine 60 mg/kg (Ketavet®). Anesthetized animals were tracheostomized to facilitate spontaneous breathing and PE-catheters were inserted into the left Arteria carotis and the right internal Vena jugularis. Body temperature was maintained constant at 37°C. NaCl 0.9% (5 ml/kg/h) was infused via the venous catheter until 180 min post lipopolysaccharide injection. At 180 min, an arterial blood sample of 0.5 ml was drawn for the determination of nitrite/nitrate (NOx) levels, which were determined using the Griess assay. Immediately thereafter, BYK191023 or vehicle was injected intravenously. The loading dose of 3/10/30 µmol/kg BYK191023 was administered as a bolus followed by continuous infusion of 3/10/30 µmol/kg/h in a volume of 1.0 ml/kg and 5.0 ml/kg/h, respectively. At 270 and 360 min post lipopolysaccharide challenge (i.e. 90 and 180 min after start of treatment), additional blood samples were drawn for NOx

determination. The inhibition of NOx increase from 180 to 360 min by BYK191023 was calculated individually for each animal (1 – (ΔNOx (treated animal) / mean ΔNOx (lipopolysaccharide/vehicle))) and expressed as % mean inhibition for each treatment group.

Prevention of lipopolysaccharide-induced Late Hypotension in Rats

Rats were anesthetized and instrumented as described before. In addition, a laparatomy was performed and the bladder was catheterized for collection of urine. Animals were mechanically ventilated with ambient air (VSM Ventilation Sequencer Module Type 698, Hugo Sachs Elektronik, March-Hugstetten, Germany) at a frequency of 70 min^{-1} and an inspiration cycle of 20%. Tidal volume was adjusted individually to yield a PCO_2 of approximately 40 mmHg (ABL™ 700, Radiometer, Willich, Germany). Blood pressure and heart rate were recorded continuously with a pressure transducer coupled to the arterial catheter. Automatic calculation of heart rate and mean arterial pressure (MAP) was done with the program NOTOCORD-Hem™ (NOTOCORD®, Croissy sur Seine, France). After a 20 min equilibration phase, lipopolysaccharide was infused via the venous catheter at a dose of 16 mg/kg/h for 1 hr (2 ml/kg/h), followed by infusion of NaCl 0.9% (5 ml/kg/h) for 2 hrs. Non-endotoxaemic control animals received physiological saline instead of lipopolysaccharide. At 180 min after start of lipopolysaccharide infusion, an arterial blood sample of 0.5 ml was drawn for determination of NOx plasma levels and animals were randomized to treatment. BYK191023 or vehicle was injected as an i.v. loading dose (50 µmol/kg in 1 ml/kg), followed by continuous i.v. infusion (50 µmol/kg/h in 5 ml/kg/h) for 3 hrs until 360 min.

Determination of Vascular Responsiveness in lipopolysaccharide Challenged Rats

Conscious rats were injected i.v. with lipopolysaccharide (2 mg/kg in 1 ml/kg) via the tail vein. After anaesthesia (thiopental and ketamine, as described before), a tracheotomy was performed, catheters were inserted into the Arteria carotis, the Vena jugularis and the Vena femoralis and NaCl 0.9% was administered as a continuous infusion simultaneously via both

venous catheters (2.5 ml/kg/h per catheter, i.e. 5 ml/kg/h total infusion volume) until t = 180 min. At t = 180 min, animals received a bolus injection of either test compound or vehicle (1 ml/kg), followed by continuous infusion of test compound or vehicle (2.5 ml/kg/h)) via the Vena jugularis (BYK191023 or vehicle) and via the Vena femoralis (L-arginine or vehicle). From 330 min to 380 min, pressor responses to subsequent i.v. bolus injections of increasing doses of norepinephrine (0.3, 1, 3 nmol/kg in 1 ml/kg) and sodium nitroprusside (3, 10, 30 nmol/kg in 1 ml/kg) were recorded. MAP was averaged for 1 min intervals and maximum change versus baseline within 10 min was calculated. Details on the treatment groups are depicted in Table 1.

Determination of Plasma Nitrite/Nitrate Levels

Nitrite/nitrate levels in plasma were determined with the Griess assay. In brief, immediately after arterial blood sampling, the samples were centrifuged (6,000 g, 4°C) for 10 min, the resulting supernatant was diluted 1:5 in deionized water and transferred to microfilter cups (Ultrafree®-MC Centrifugal Filter Units, 10 kD, Millipore GmbH, Schwalbach, Germany). After further centrifugation of the microfilter cups for 60 min to get rid of haemoglobin, the NOx concentration of the resulting filtrate solution was determined with a commercially available Griess assay test kit (Total Nitric Oxide Assay Kit, Assay Designs, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Statistics

Data are presented as means ± standard error of the mean (SEM). Statistical differences were determined by Tukey's Multiple Comparison test or by Bonferroni's Multiple Comparison test of selected groups after One-Way ANOVA or Two-Way ANOVA, as indicated. In case of unequal variances (Bartlett's test, $p < 0.05$), data were log transformed before analysis. If log-transformation failed to equalize variances, the Kruskal-Wallis test, followed by Dunn's test

was used. A value of $p < 0.05$ was considered significant. All tests were performed with GraphPad Prism, version 4.03 for Windows (GraphPad, San Diego, CA).

Materials

BYK191023 was synthesized at ALTANA Pharma (Konstanz, Germany). lipopolysaccharide (Salmonella typhosa), (-)-norepinephrine bitartrate salt hydrate, L-arginine hydrochloride, sodium nitroprusside dehydrate and hydroxylamine hydrochloride were purchased from Sigma (Deisenhofen, Germany), polyethylenglycol 400 was from Serva (Heidelberg, Germany), Ketavet® was obtained from Pharmacia & Upjohn (Erlangen, Germany), NaCl 0.9% was supplied by B. Braun (Melsungen, Germany) and Trapanal® was from ALTANA Pharma.

Results

Effect of BYK191023 on Plasma NOx Levels in Lipopolysaccharide-Challenged Rats

Intravenous lipopolysaccharide administration induced iNOS activity, indicated by an 4-5 fold increase in plasma NOx levels in lipopolysaccharide-challenged animals at the time of treatment start (180 min after lipopolysaccharide) (Fig. 1). Administration of BYK191023 as an intravenous loading dose, followed by continuous intravenous infusion dose-dependently reduced the steady accumulation of plasma NOx measured at 270 min and at 360 min post lipopolysaccharide challenge. BYK191023 inhibited the rise in plasma NOx levels from 180 to 360 min post lipopolysaccharide by 23 ± 16% (3 µmol/kg/h n = 7), 36 ± 13% (10 µmol/kg/h n = 8) and 68 ± 9% (30 µmol/kg/h), n = 8), resulting in an ED_{50} of 14.9 µmol/kg/h. Steady-state plasma concentrations of BYK191023 ranged from 1 µM (3 µmol/kg/h) to 25 µM (30 µmol/kg/h) and were reached after approximately 60-90 min of infusion, reflecting the short half-life of the compound in the rat ($t_{1/2} \approx 0.5$ h).

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Effect of BYK191023 on Lipopolysaccharide-induced Hypotension in Anaesthetized Rats

We assessed whether inhibition of plasma NOx increase with BYK191023 counteracted lipopolysaccharide-induced delayed hypotension in anesthetized rats. To provide substantial inhibition of plasma NOx increase, BYK191023 was administered at a dose of 50 µmol/kg/h (approximately threefold the ED_{50} determined in the previously described model). Whereas blood pressure remained relatively stable in saline treated control animals, infusion of lipopolysaccharide resulted in an early biphasic decrease in MAP within the first hour after challenge, followed by a transient normalization of MAP at 2-3 hrs after challenge (Fig. 2). A second phase of progressive hypotension became evident after approximately 3-4 hrs in lipopolysaccharide-challenged and vehicle-treated rats, leading to a 20 mmHg decrease in MAP by the end of the experimental period. Treatment of endotoxaemic rats with BYK191023 completely prevented development of this delayed hypotension. At the end of the 6 h observation period, MAP in BYK191023 treated animals was indistinguishable from that of saline treated control rats (saline/vehicle: 94 ± 15 mmHg, n = 10; lipopolysaccharide/vehicle: 76 ± 17 mmHg, n = 13; lipopolysaccharide/BYK191023: 96 ± 21 mmHg, n = 10 (mean ± SD)) (Fig. 2). To account for differences in pre-treatment MAP values between the individual animals, the MAP from 160-175 min was used as a baseline to demonstrate the absolute change in blood pressure (as mmHg) after initiation of therapeutic treatment and to calculate the mean MAP change over the 180 min treatment phase. Whereas in non-endotoxaemic control animals MAP slightly decreased by 7 ± 10 mmHg (mean ± SD), lipopolysaccharide-challenged animals displayed a mean reduction of 18 ± 6 mmHg. Treatment with BYK191023 completely abrogated the lipopolysaccharide-dependent deterioration of MAP, demonstrating mean MAP changes equal to lipopolysaccharide-untreated control animals (mean MAP reduction: 6 ± 10 mmHg, $p < 0.05$ vs. lipopolysaccharide/vehicle).

Determination of plasma NOx values from samples drawn at 180 min and 360 min demonstrated an 83% inhibition of NOx increase in BYK191023 treated animals as compared to the lipopolysaccharide/vehicle group ($p < 0.01$). In line with our previous data, no increase

in basal NOx levels (28 ± 14 µM at 180 min vs. 25 ± 10 µM at 360 min) was found in saline treated control animals.

Effect of BYK191023 on Blood Pressure of Non-Endotoxaemic Anaesthetized Rats

To exclude the possibility that despite the high *in vitro* selectivity of BYK191023 for iNOS vs. eNOS/nNOS (Strub et al., 2005), the observed stabilization of MAP in endotoxaemic rats was due to inhibition of constitutively expressed NOS, we investigated the effect of BYK191023 on MAP in saline treated animals. As depicted in Fig. 3, blood pressure in non-septic, saline challenged animals treated with BYK191023 did not differ from that in vehicle treated controls, arguing against any iNOS-independent vasopressor activity of BYK191023.

Effect of BYK191023 on Lipopolysaccharide-induced Vascular Hyporesponsiveness

Besides systemic hypotension, lipopolysaccharide-challenged animals frequently develop vascular hyporesponsiveness to administration of vasopressor agents. We addressed the role of iNOS derived nitric oxide in lipopolysaccharide-induced vasoplegia by assessing the vasopressor effect of norepinephrine in lipopolysaccharide-challenged rats. Repeated injection of norepinephrine at doses of 0.3, 1, and 3 nmol/kg, resulted in a dose-dependent transient increase in MAP in all animals (Fig. 4a). However, the pressor response to norepinephrine injection was significantly depressed in endotoxaemic rats as compared to non-septic controls. Administration of BYK191023 (50 µmol/kg + 50 µmol/kg/h) prior to norepinephrine injection attenuated the depression of vascular responsiveness associated with lipopolysaccharide challenge. The restoration of norepinephrine responsiveness by BYK191023 consisted in reversal of hyporeactivity by 10 ± 27%, 23 ± 18%, and 42 ± 20% for the different vasopressor doses 0.3, 1, and 3 nmol/kg, respectively, with the most pronounced effect at the highest norepinephrine dose (Fig. 4a).

To assess whether vascular hyporesponsiveness in lipopolysaccharide-challenged animals was limited to suppression of vasopressor responses to alpha-adrenergic receptor stimulation or rather represented a general stimulus-independent phenomenon, we subjected the animals to additional injections of the endothelium-independent vasodilator sodium nitroprusside. In line with impaired norepinephrine pressor responses, endotoxaemic rats displayed a significantly diminished depressor response compared to control rats after repeated stimulation with increasing doses of sodium nitroprusside (Fig. 4b). Treatment with BYK191023 partially reversed vascular sodium nitroprusside hyporesponsiveness by 33 ± 32% (3 nmol/kg sodium nitroprusside dose), 49 ± 22% (10 nmol/kg), and 41 ± 20% (30 nmol/kg), respectively.

To substantiate the view that the beneficial effects of BYK191023 were mediated via inhibition of nitric oxide release from iNOS, we included a treatment group that in addition to BYK191023 (50 µmol/kg + 50 µmol/kg/h) received a ten-fold excess of the NOS substrate L-arginine (500 µmol/kg + 500 µmol/kg/h). Since BYK191023 has been shown to be an L-arginine-competitive inhibitor of iNOS (Strub et al., 2005), co-infusion of excess L-arginine should counteract the inhibitory effect of BYK191023 on NO production and thus reverse its beneficial effects on vascular hyporesponsiveness. Reversal of BYK191023-mediated iNOS inhibition by excess L-arginine was verified by measuring the increase in plasma NOx levels after treatment (Fig. 5). Whereas co-infusion of BYK191023 with vehicle yielded a 91 ± 7% inhibition of the lipopolysaccharide-induced NOx increase, the extent of NOx inhibition was significantly reduced (56 ± 14%; $p < 0.05$) in animals receiving an additional infusion of L-arginine, indicating a significant albeit only partial reversal of BYK191023 mediated iNOS inhibition. Concerning vascular responsiveness, co-infusion of L-arginine completely abrogated the beneficial effects of BYK191023 on vasopressor responses to norepinephrine, whereas the restoration of sodium nitroprusside responsiveness conferred by iNOS inhibition was only partially reversed. (Fig. 4a and 4b).

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Discussion

A large number of studies in animal models of acute and chronic inflammation have demonstrated beneficial effects of pharmacological intervention with nitric oxide production and activity or genetic deletion of the iNOS gene, suggesting that inhibition of iNOS could represent an interesting novel therapeutic principle (Cuzzocrea et al., 2001; Cuzzocrea et al., 2002; Ichinose et al., 2003; MacMicking et al., 1995; Matejovic et al., 2004). During severe sepsis, uncontrolled excess synthesis and release of nitric oxide by iNOS is considered a potential contributor to pathological vasodilatation, eventually leading to severe hypotension with insufficient organ perfusion and consequently multi-organ failure (Titheradge, 1999; Cobb, 2001). Although clinical trials with the non-specific NOS inhibitor L-NMMA in patients with septic shock yielded unfavourable results (Bakker et al., 2004; Lopez et al., 2004), inhibition of iNOS-derived nitric oxide by selective drugs is still considered an interesting target for novel therapeutic agents in septic shock (Ichinose et al., 2003).

In the present study, we characterized the *in vivo* effects of BYK191023, a novel and potent iNOS inhibitor with an excellent isoform selectivity profile versus eNOS (>1000-fold) and nNOS (200-fold) (Strub et al., 2005), on delayed hypotension and vascular hyporesponsiveness conferred by systemic lipopolysaccharide administration to rats. Injection of lipopolysaccharide is commonly employed to induce iNOS in animal models, resulting in massive synthesis and release of NO, which can indirectly be measured by determination of plasma NOx levels (Tracey et al., 1995). The substantial increase in plasma NOx upon lipopolysaccharide challenge is considered to be due to induction of iNOS, since no changes in plasma NOx levels were observed in lipopolysaccharide-challenged iNOS gene deficient mice (Laubach et al., 1995; MacMicking et al., 1995). In the curative treatment setting employed in our studies, i.e. start of treatment at 3 hrs after lipopolysaccharide, infusion of BYK191023 dose-dependently attenuated the increase in plasma NOx levels associated with lipopolysaccharide challenge with an ED_{50} of 14.9 µmol/kg/h (Fig. 1), demonstrating that in contrast to inhibitors of iNOS induction or iNOS dimerization (Ichinose et al., 2003), BYK191023 acts via inhibition of already induced and active iNOS. This conclusion is supported by our experiments on reversal of NOx inhibition by additional L-

arginine substitution (Fig. 5) as well as by data from in vitro studies demonstrating arginine-competitive inhibition of iNOS activity rather than interference with iNOS expression or iNOS dimerization (Strub et al., 2005). The in vivo potency of BYK191023 was comparable to that of L-NMMA (ED_{50} = 11.1 µmol/kg/h), but lower than that of N-(3-(amino methyl)benzyl)acetamidine (1400W; ED_{50} = 2.3 µmol/kg/h) under our model-specific conditions, reflecting the relatively high clearance of BYK191023 in the rat. We then studied whether reduction of plasma NOx levels by BYK191023 treatment translated into a more clinically relevant parameter, i.e. prevention of lipopolysaccharide-induced hypotension. High dose lipopolysaccharide infusion resulted in an early biphasic fall in MAP within the first hour, followed by gradual normalization of MAP values by 2-3 h after start of lipopolysaccharide infusion and a steady decrease in MAP from 3-6 h. Continuous administration of BYK191023 starting at 3 h after lipopolysaccharide challenge completely prevented the development of systemic hypotension observed in the vehicle treated endotoxaemic animals and maintained MAP at levels indistinguishable from those of saline challenged controls. These data are in line with a number of studies demonstrating prevention of hypotension or restoration of normal blood pressure in lipopolysaccharide-challenged animals by pharmacological interference with nitric oxide production. However, many studies employed either non- or poorly iNOS-selective NOS inhibitors such as N^G-nitro-L-arginine methyl ester (L-NAME) (Filep et al., 1997; Strunk et al., 2001), L-NMMA (Gray et al., 1991; Zhang et al., 1997), N^6-(1-iminoethyl)-L-lysine (L-NIL) (Schwartz et al., 2001; Strunk et al., 2001), or aminoguanidine (Ruetten et al., 1996; Wu et al., 1995), the latter exhibiting additional activities apart from NOS inhibition (Picard et al., 1992). Consequently, it has been difficult to depict whether the reported anti-hypotensive effects stemmed from reversal of pathological iNOS-mediated vasodilatation or were merely secondary to vasoconstriction via interference with physiological eNOS activity. To verify that the observed counteraction of lipopolysaccharide-induced hypotension was indeed due to iNOS inhibition and not mediated via co-inhibition of constitutive NOS activity, the effect of BYK191023 on systemic blood pressure in saline challenged rats was studied. In contrast to similar experiments with the non-selective compound L-NMMA (data not shown), administration of BYK191023 at a dose that prevented hypotension in lipopolysaccharide-challenged rats did not exert any MAP increase in non-

endotoxaemic control animals, arguing against a major involvement of constitutive NOS inhibition in the observed beneficial effects in endotoxaemic animals (Fig. 3). These results confirm data from infusion studies with the selective iNOS inhibitors 1400W (Cheng et al., 2003; Wray et al., 1998) and 3-[[2-[(1-iminoethyl)amino]ethyl]sulphonyl]-L-alanine (GW273629) (Alderton et al., 2005) and corroborate the view that excessive NO production by iNOS is a major mechanism underlying the development of hypotension in the course of systemic inflammation. According to our data, selective inhibition of iNOS by BYK191023 without interfering with constitutively expressed e/nNOS activity should be sufficient for the desired therapeutic effect on macrocirculation.

In studies with volunteers as well as in animal models of systemic inflammation, challenge with lipopolysaccharide or immune stimuli from Gram-positive bacteria rapidly confers vascular hyporesponsiveness to exogenous catecholamine therapy (Pleiner et al., 2003; Ruetten et al., 1996). Clinically, desensitization of the large resistance vessels to vasoconstrictor administration is a critical event associated with an increased risk of morbidity in septic shock patients depending on exogenous catecholamine therapy for maintaining an adequate blood pressure level.

We were interested whether selective iNOS inhibition by BYK191023 at a dose that effectively abolished lipopolysaccharide-induced delayed hypotension also reversed vascular hyporesponsiveness, as reported for several less selective iNOS inhibitors (Cai et al., 1996; Gray et al., 1991; Wu et al., 1995). In our studies, pressor responses to norepinephrine were severely compromised in endotoxaemic animals as compared to saline treated controls when tested at 5-6 h after challenge. In addition, lipopolysaccharide-challenged animals displayed a significantly impaired blood pressure response to injection of the endothelium independent vasodilator sodium nitroprusside, indicating lipopolysaccharide-induced stimulus-independent vasoplegia rather than a selective desensitization to alpha adrenergic agonists. Administration of BYK191023 at 3 h after lipopolysaccharide challenge improved the compromised pressor and depressor responses of lipopolysaccharide-challenged animals to stimulation with norepinephrine and sodium nitroprusside, respectively. This partial restoration of normal responsiveness was due to competitive inhibition of the L-arginine/ nitric oxide pathway and

not related to other unidentified effects of BYK191023, since co-administration of a ten-fold excess of the substrate L-arginine largely reversed the beneficial effects of BYK191023 treatment. The improvement of depressor responses to sodium nitroprusside was less efficiently reversed by L-arginine co-administration than the positive effect of BYK191023 therapy on pressor responses to norepinephrine (Fig. 4b and 4a) providing evidence for a higher susceptibility to iNOS-mediated desensitization of alpha-adrenergic vasoconstriction as compared to soluble guanylate cyclase-dependent vasodilation. Treatment with BYK191023 only partially restored normal vascular responsiveness to norepinephrine and sodium nitroprusside despite nearly complete suppression of plasma NOx levels, suggesting additional pathways of vascular desensitization inaccessible to iNOS inhibitor therapy. However, we employed a curative administration schedule, where iNOS inhibition therapy was initiated at a time when plasma NOx levels where already significantly increased. Since it has been demonstrated that the induction, but not necessarily the maintenance of lipopolysaccharide-induced long-term vasoplegia depended on NOS activity (Silva-Santos et al., 2002), it seems feasible that in our model the therapeutic treatment phase of 2-3 h prior to determination of the vascular responsiveness was too short to allow recovery of already desensitized vessels and the beneficial effect of BYK191023 merely reflected prevention of further vascular damage. Thus, a prolongation of BYK191023 therapy could result in a more pronounced restoration of vascular responsiveness as compared to the acute reversal of hyporesponsiveness observed in our current experimental setting.

The actual mechanism responsible for the prevention or reversal of inflammation-induced hypotension by selective iNOS inhibition has not been definitely identified, yet. Based on our data, it seems reasonable to propose that inhibition of excess NO release from iNOS by BYK191023 normalized vascular tone and blood pressure in endotoxic shock either directly via counteraction of the NO-mediated pathological vasodilatation, or indirectly by restoring vascular responsiveness to endogenous vasoconstrictors.

Although future studies in large animal models of sepsis as well as in septic shock patients are warranted to demonstrate whether normalization of vascular resistance by selective iNOS inhibition beneficially affects morbidity and mortality, reversal of pathologic hypotension by

BYK191023 in the present rodent endotoxin model holds promise for a potential clinical benefit of this novel class of short acting and highly selective iNOS inhibitors.

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Footnotes

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Figure legends

Fig. 1. Effect of BYK191023 on lipopolysaccharide-induced increase in plasma NOx levels. NaCl 0.9% or lipopolysaccharide (0.5 mg/kg i.v.) was injected at time zero and BYK191023 was administered as an i.v. bolus at 180 min, followed by continuous i.v. infusion from 180 to 360 min post lipopolysaccharide. Plasma was sampled at 180, 270 and 360 min. Data represent means ± SEM for 7-10 animals / group.

Fig. 2. Effect of BYK191023 on mean arterial blood pressure (MAP) in lipopolysaccharide-challenged rats. Animals received an i.v. infusion of NaCl 0.9% or lipopolysaccharide (16 mg/kg/h) for one hour starting at time zero, followed by resuscitation with NaCl 0.9% for further 2 hours until t = 180 min. BYK191023 or vehicle was administered as an i.v. bolus injection (50 µmol/kg) at 180 min, followed by continuous i.v. infusion (50 µmol/kg/h) from 180 to 360 min. MAP was recorded continuously. Blood samples of 0.5 ml were drawn at 180 and 360 min. Mean values ± SEM are presented at 5 min intervals for 10-13 animals / group.

Fig. 3. Effect of BYK191023 on mean arterial blood pressure (MAP) in naive rats. Animals received an i.v. infusion of NaCl 0.9% three hours starting at time zero. BYK191023 or vehicle was administered as an i.v. bolus injection (50 µmol/kg) at 180 min, followed by continuous i.v. infusion (50 µmol/kg/h) from 180 min to 360 min. MAP was recorded continuously. Blood samples of 0.5 ml were drawn at 180 and 360 min. Mean values ± SEM are presented at 5 min intervals for 10 animals / group.

Fig. 4. Effect of BYK191023 on vascular hyporesponsiveness in lipopolysaccharide-challenged rats. Animals received NaCl 0.9% or lipopolysaccharide (2 mg/kg) at time zero. At 180 min, vehicle, BYK191023 (50 µmol/kg + 50 µmol/kg/h) or L-arginine (500 µmol/kg + 500 µmol/kg/h) were administered via i.v. bolus injection, followed by i.v. continuous infusion from 180 to 360 min. From 300 to 390 min, pressor and depressor responses to injection of increasing doses of norepinephrine (NE) (4A) and sodium nitroprusside (SNP) (4B),

respectively, were recorded. Maximal blood pressure increase (NE) or decrease (SNP) was calculated and expressed as means ± SEM for 8 animals / group. * $p < 0.05$ vs. lipopolysaccharide/vehicle, ** $p < 0.01$ vs. lipopolysaccharide/vehicle, *** $p < 0.001$ vs. lipopolysaccharide/vehicle (Two-Way ANOVA with Bonferroni's posttest).

Fig. 5. Effect of L-arginine administration on inhibition of plasma NOx increase by BYK191023 in lipopolysaccharide-challenged rats. Animals were injected with NaCl 0.9% or lipopolysaccharide (2 mg/kg) at time zero. At 180 min, vehicle, BYK191023 (50 µmol/kg + 50 µmol/kg/h) or L-arginine (500 µmol/kg + 500 µmol/kg/h) were administered via i.v. bolus injection, followed by continuous i.v. infusion from 180 to 360 min. From 300 to 390 min, pressor and depressor responses to injection of increasing doses of norepinephrine and sodium nitroprusside, respectively, were recorded. Plasma NOx was determined in samples drawn at 180 min (i.e. prior to BYK191023 treatment) and at 390 min from which absolute changes in plasma NOx levels from 180 to 390 min were calculated. Data are expressed as means ± SEM for 7-8 animals / group. *** $p < 0.001$ as calculated by One-Way ANOVA, followed by Bonferroni's Multiple Comparison Test.

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Tables

Table 1: Treatment groups employed for testing of vascular hyporesponsiveness induced by lipopolysaccharide (LPS) challenge

Group	**1**	**2**	**3**	**4**
Challenge	NaCl 0.9%	LPS 2 mg/kg	LPS 2 mg/kg	LPS 2 mg/kg
Treatment (V. jugularis)	Vehicle [a]	Vehicle	BYK191023 [b]	BYK191023
Treatment (V. femoralis)	NaCl 0.9%	NaCl 0.9%	NaCl 0.9%	L-arginine [c]

[a] Vehicle consisted of NaCl 0.9% containing PEG 400 2.5%

[b] BYK191023 was administered i.v. as a 50 µmol/kg bolus injection followed by 50 µmol/kg/h continuous infusion

[c] L-arginine was administered i.v. as a 500 µmol/kg bolus injection followed by 500 µmol/kg/h continuous infusion

Figure 1

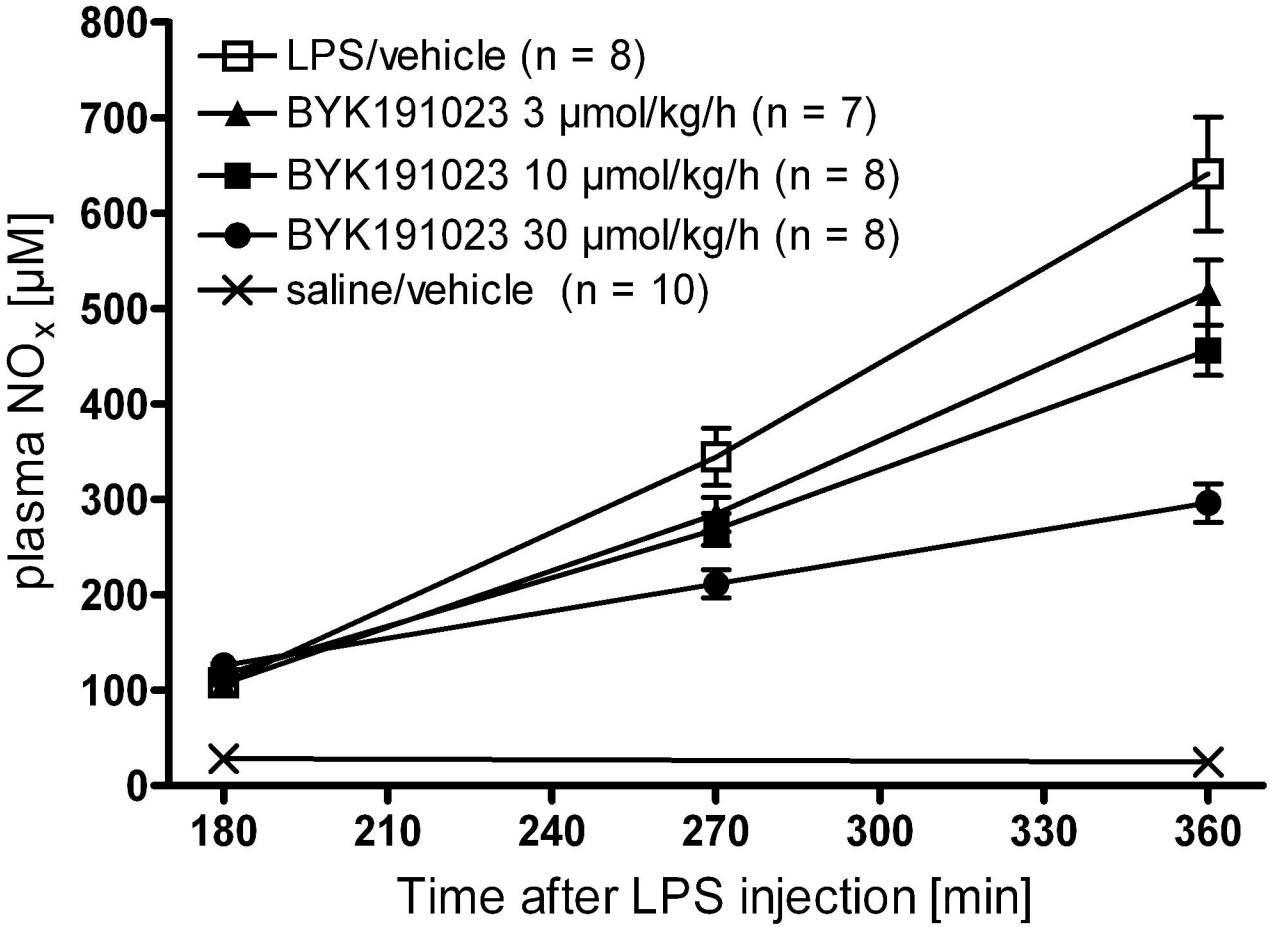

LPS/vehicle (n = 8)
BYK191023 3 µmol/kg/h (n = 7)
BYK191023 10 µmol/kg/h (n = 8)
BYK191023 30 µmol/kg/h (n = 8)
saline/vehicle (n = 10)
plasma NOx [µM]
800
700
600
500
400
300
200
100
0
180
210
240
270
300
330
360
Time after LPS injection [min]

Figure 2

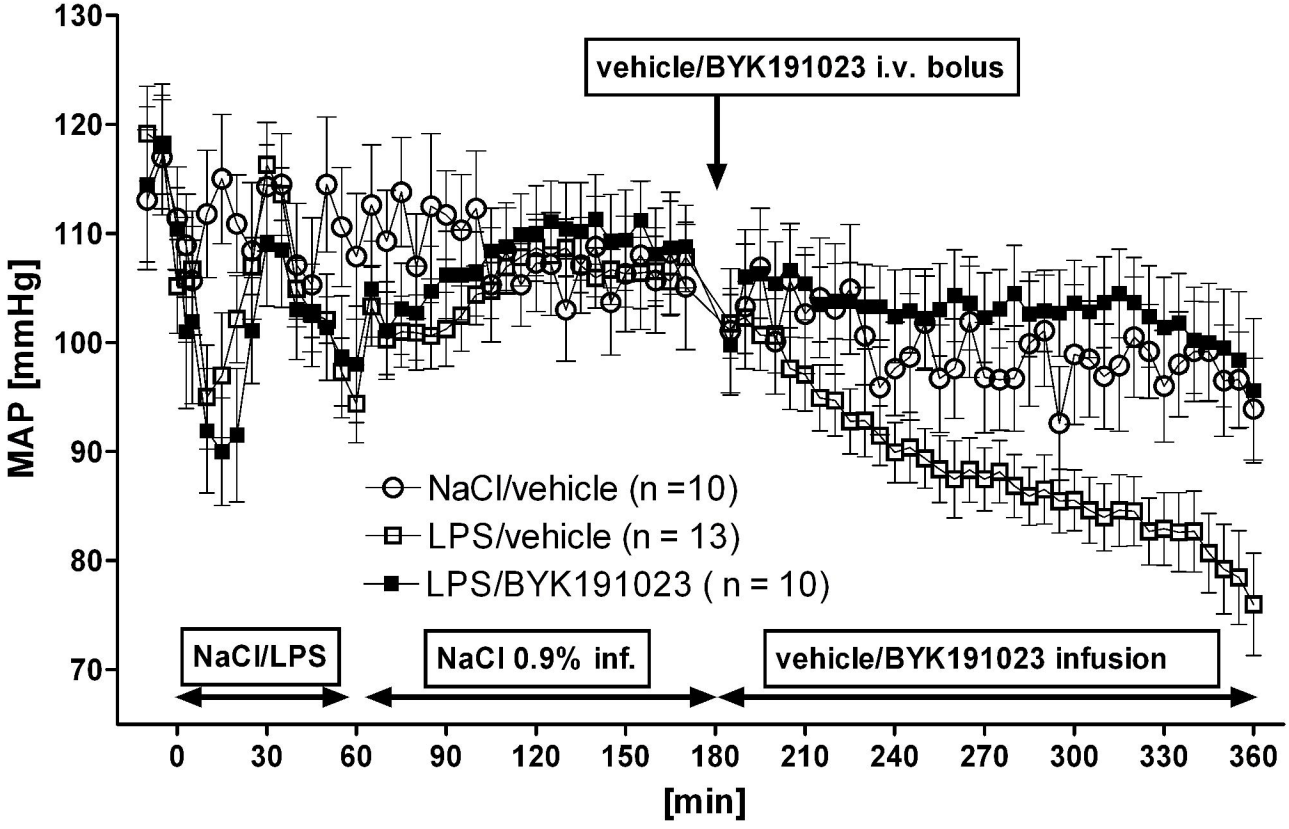

130
120
110
100
90
80
70
MAP [mmHg]
vehicle/BYK191023 i.v. bolus
NaCl/vehicle (n =10)
LPS/vehicle (n = 13)
LPS/BYK191023 (n = 10)
NaCl/LPS
NaCl 0.9% inf.
vehicle/BYK191023 infusion
0
30
60
90
120
150
180
210
240
270
300
330
360
[min]

Figure 3

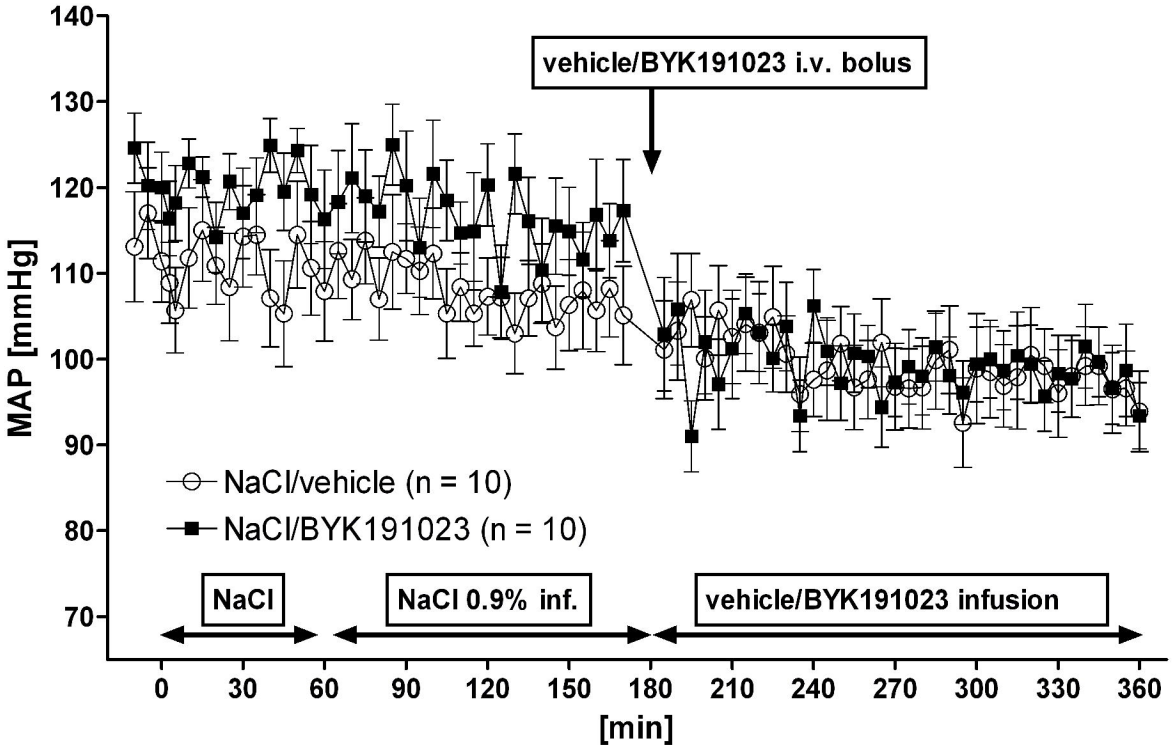

140
130
120
110
100
90
80
70
MAP [mmHg]
vehicle/BYK191023 i.v. bolus
NaCl/vehicle (n = 10)
NaCl/BYK191023 (n = 10)
NaCl
NaCl 0.9% inf.
vehicle/BYK191023 infusion
0
30
60
90
120
150
180
210
240
270
300
330
360
[min]

Figure 4

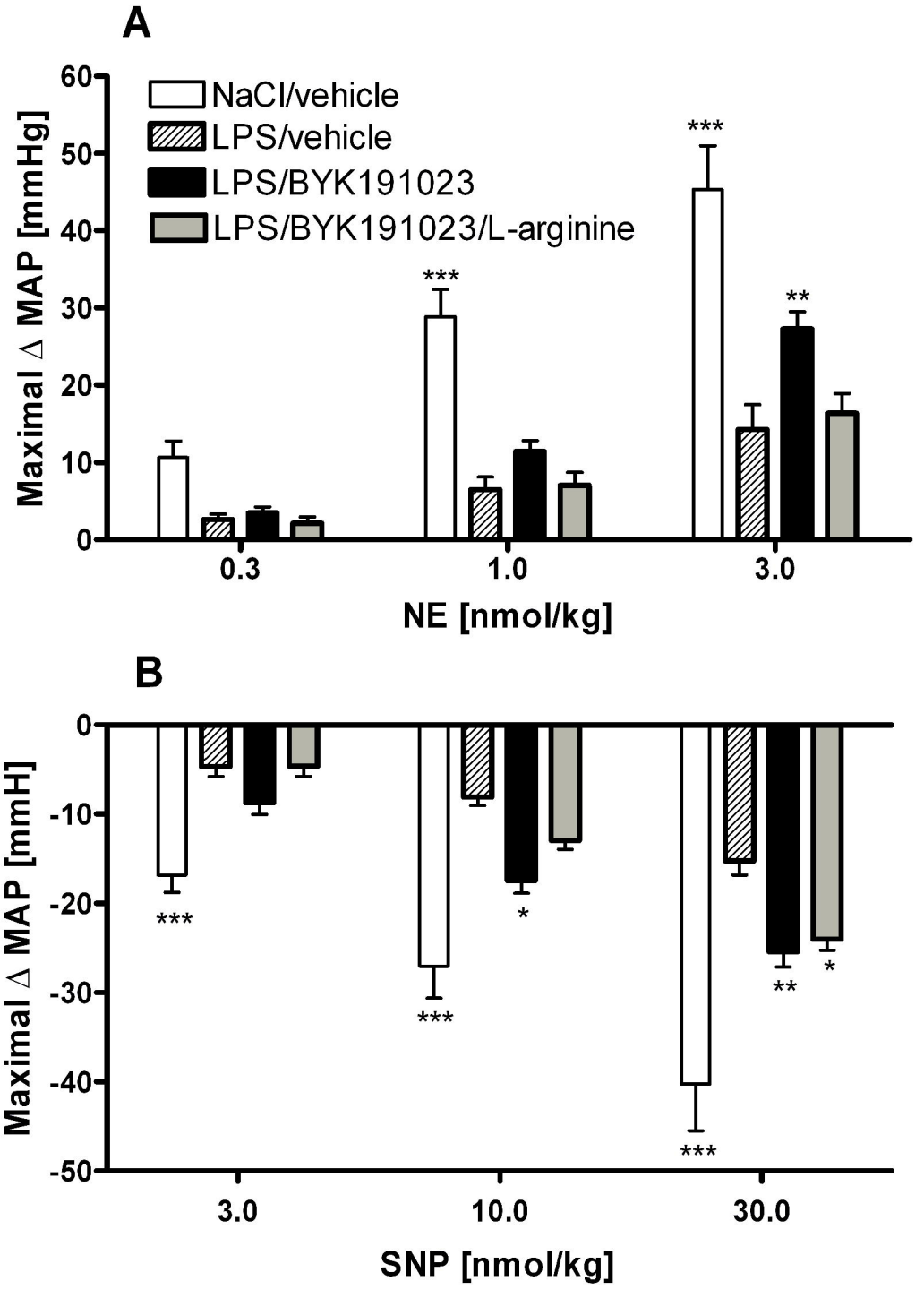
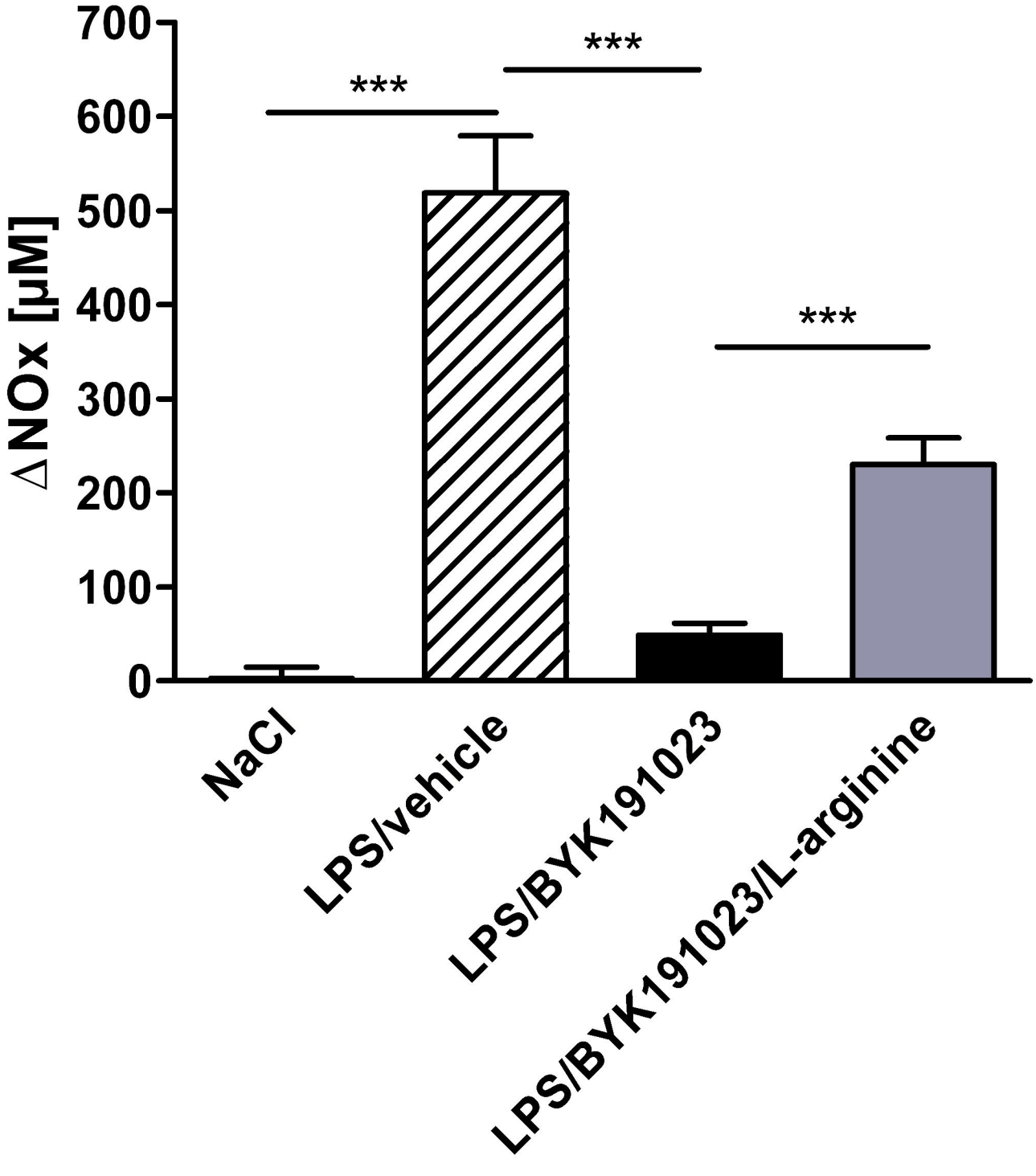

A
NaCl/vehicle
LPS/vehicle
LPS/BYK191023
LPS/BYK191023/L-arginine
Maximal Δ MAP [mmHg]
60
50
40
30
20
10
0
***
***
**
0.3
1.0
3.0
NE [nmol/kg]
B
Maximal Δ MAP [mmH]
0
-10
-20
-30
-40
-50
***
***
*
***
**
*
3.0
10.0
30.0
SNP [nmol/kg]

Figure 5


700
600
500
400
300
200
100
0
ΔNOx [µM]
***
***
***
NaCl
LPS/vehicle
LPS/BYK191023
LPS/BYK191023/L-arginine