Vasorelaxing Effect of BAY 41-2272 in Rat Basilar Artery Involvement of cGMP-Dependent and Independent Mechanisms

Cleber E. Teixeira, Fernanda B.M. Priviero, Joseph Todd Jr, R. Clinton Webb

Abstract—Decreases in intrinsic NO cause cerebral vasospasms because of the dysregulation of cGMP formation by NO-mediated pathways. Because 5-cyclopropyl-2-[(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272) is a potent soluble guanylyl cyclase (sGC) stimulator in an NO-independent manner, this study aimed to investigate the mechanisms underlying the relaxant effects of BAY 41-2272 in the rat basilar artery. BAY 41-2272 (0.0001 to 1 μmol/L) induced relaxations in a concentration-dependent manner, with pEC_50 values of 8.13±0.03 and 7.63±0.05 in intact and denuded rings, respectively. The sGC inhibitor 1H-[1,2,4] oxadiazolo [4,3-a]quinolin-1-one (ODQ) markedly displaced the curve for BAY 41-2272 to the right in intact or denuded rings (∼10-fold). The NO synthesis inhibitor N^0-nitro-l-arginine methyl ester caused a rightward shift in the curve for BAY 41-2272 (4-fold), whereas the phosphodiesterase type 5 inhibitor sildenafil enhanced BAY 41-2272–induced relaxations (3- to 4-fold). The Na^+-K^+-ATPase inhibitor ouabain caused 3-fold rightward shifts in the curves for BAY 41-2272. Ca^2+-induced contractions in K^+ depolarized rings were significantly attenuated by BAY 41-2272 in an ODQ-insensitive manner. The NO donor glycercyl trinitrate and BAY 41-2272 caused rightward shifts in the contractile responses to serotonin. Their coincubation caused a synergistic inhibition of serotonin-induced contractions. BAY 41-2272 and glycercyl trinitrate increased cGMP levels (but not cAMP) by 10-fold and 4-fold above baseline, respectively, in an ODQ-sensitive manner. cGMP levels increased by 50-fold after coincubation. BAY 41-2272 potently relaxes the rat basilar artery in a synergistic fashion with NO. Targeting the sGC with selective activators, such as BAY 41-2272, may represent a new therapy to treat cerebrovascular disease. (Hypertension. 2006;47[part 2]:596-602.)

Key Words: nitric oxide ■ cyclic GMP ■ nitric oxide synthase

Nitric oxide (NO) derived from perivascular nerve fibers and endothelial cells acts as vasodilator for the smooth muscle in the wall of cerebral arteries and, hence, plays an essential role in the regulation of cerebral blood flow under physiological and pathological conditions. Physiologically, NO is produced by NO synthase (NOS) present in the endothelium (eNOS) and nerves (nNOS) to maintain a basal vasorelaxation, whereas the phosphodiesterase type 5 inhibitor sildenafil enhances NO-mediated pathways. Because 5-cyclopropyl-2-[(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272) is a potent soluble guanylyl cyclase (sGC) stimulator in an NO-independent manner, this study aimed to investigate the mechanisms underlying the relaxant effects of BAY 41-2272 in the rat basilar artery. BAY 41-2272 (0.0001 to 1 μmol/L) induced relaxations in a concentration-dependent manner, with pEC_50 values of 8.13±0.03 and 7.63±0.05 in intact and denuded rings, respectively. The sGC inhibitor 1H-[1,2,4] oxadiazolo [4,3-a]quinolin-1-one (ODQ) markedly displaced the curve for BAY 41-2272 to the right in intact or denuded rings (∼10-fold). The NO synthesis inhibitor N^0-nitro-l-arginine methyl ester caused a rightward shift in the curve for BAY 41-2272 (4-fold), whereas the phosphodiesterase type 5 inhibitor sildenafil enhanced BAY 41-2272–induced relaxations (3- to 4-fold). The Na^+-K^+-ATPase inhibitor ouabain caused 3-fold rightward shifts in the curves for BAY 41-2272. Ca^2+-induced contractions in K^+ depolarized rings were significantly attenuated by BAY 41-2272 in an ODQ-insensitive manner. The NO donor glycercyl trinitrate and BAY 41-2272 caused rightward shifts in the contractile responses to serotonin. Their coincubation caused a synergistic inhibition of serotonin-induced contractions. BAY 41-2272 and glycercyl trinitrate increased cGMP levels (but not cAMP) by 10-fold and 4-fold above baseline, respectively, in an ODQ-sensitive manner. cGMP levels increased by 50-fold after coincubation. BAY 41-2272 potently relaxes the rat basilar artery in a synergistic fashion with NO. Targeting the sGC with selective activators, such as BAY 41-2272, may represent a new therapy to treat cerebrovascular disease. (Hypertension. 2006;47[part 2]:596-602.)

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Methods

Experiments were conducted in accordance with institutional guidelines and approved by the Medical College of Georgia Institutional Animal Care and Use Committee. Experiments were performed on male Sprague-Dawley rats (250 to 300 g) obtained from Harlan Laboratories (Indianapolis, IN). Animals were housed 2 per cage on a 12-hour light–dark cycle and fed a standard chow diet with water ad libitum.

Vascular Reactivity Studies

The animals were stunned by inhalation of CO2, euthanized by decapitation, and exsanguinated. The brain was quickly removed and placed in chilled Krebs–Henseleit buffer of the following composition (mmol/L): NaCl, 130; NaHCO3, 14.9; dextrose, 5.5; KCl, 4.7; KH2PO4, 1.18; MgSO4·7H2O, 1.17; and CaCl2·2H2O, 1.6. The basilar artery was excised, cleaned of connective tissue under a dissection microscope, and divided into rings of 2 mm in length. Each ring was mounted in a wire myograph for isometric force recording (Danish Myograph Technology) coupled with a PowerLab 8/SP data acquisition system (software Chart 5.0, ADInstruments). The bathing solution was maintained at 37°C and continuously gassed with 95% O2 and 5% CO2. Tissues were allowed to equilibrate for 45 minutes under a resting tension of 3 mN. In some experiments, the endothelium was removed by gently rubbing the intimal surface with a 40-μm stainless steel wire.

After equilibration, the ability of the preparations to develop contractile responses was assessed in 80 mmol/L K+-substituted Krebs–Henseleit solution (achieved by the substitution of NaCl in Krebs buffer with an equimolar concentration of KCl). Functional removal of the endothelium was verified by the lack of relaxation to acetylcholine (ACh, 1 μmol/L) in vessels precontracted with serotonin (5-HT, 1 μmol/L). Cumulative concentration-response curves to 5-HT (0.0001 to 10 μmol/L) were constructed after the addition of a KCl (80 mmol/L)-induced contraction. All data are expressed as mean±SEM of n experiments.

Cyclic Nucleotide Levels

Brain stem slices were equilibrated for 20 minutes in warmed and oxygenated Krebs solution and then stimulated for 10 minutes with BAY 41-2272 (0.1 μmol/L), GTN (10 μmol/L), forskolin (1 μmol/L), or their combination in the absence or presence of 1H-[1,2,4] oxadiazolo[4,3-a]quinolin-1-one (ODQ; 0 μmol/L). Preparations were collected immediately by freezing the segments in liquid nitrogen. Cyclic nucleotides were extracted and quantified using commercially available kits (Cayman Chemical Cyclic GMP/CAMP EIA kit) as described previously.20

Drugs

Acetylcholine, 4-aminopyridine, apamin, charybdotoxin, forskolin, glibenclamide, N+-nitro-l-arginine methyl ester (l-NAME), ouabain, ODQ, serotonin, tetrodotoxin, and trichloroacetic acid were purchased from Sigma Chemical Co. The compounds 5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyrimidin-4-ylamine (BAY 41-2272), S-methyl-l-thiocitrulline, and 3-Br-7-nitroindazol (3-Br-7-NI) were obtained from Ax fora, LLC. Glycerol trinitrate (nitroglycerin, 5 mg/mL glass vials) was acquired from American Regent Laboratories. Sensenafil was obtained from Pfizer. BAY 41-2272, 3-Br-7-NI, ODQ, forskolin, sildenafil, and glibenclamide were prepared in dimethyl sulfoxide. The final concentration of the solvent did not exceed 0.1%. Preliminary experiments ascertained the lack of response to dimethyl sulfoxide. Other chemicals were dissolved in deionized water.

Statistical Analysis

Relaxant responses are expressed as a percentage of the level of precontraction. Contractile responses are calculated as a percentage of KCl (80 mmol/L)-induced contraction. All data are expressed as mean±SEM. The statistical significance of was calculated using 2-way ANOVA followed by Bonferroni’s post hoc test. A level of P<0.05 was considered to be statistically significant.

Results

Role of Endothelium in BAY 41-2272 Responses

As shown in Figure 1, cumulative addition of BAY 41-2272 (0.0001 to 1 μmol/L) produced sustained and concentration-dependent relaxations of endothelium-intact (n=24) and denuded (n=30) rings precontracted with 5-HT (1 μmol/L). However, BAY 41-2272–induced vasorelaxations were significantly (P<0.01) more potent in intact compared with denuded vessels, with pEC50 values of 8.15±0.03 and 7.65±0.06, respectively.

Effect of ODQ

The addition of ODQ (10 μmol/L) caused a significant contraction when the endothelium was present (27±8%). It was unnecessary to correct for this effect, because the

![Figure 1. Original traces (a) and graphic representation (b) of concentration-response curves to BAY 41-2272.](http://hyper.ahajournals.org/)
steady-state 5-HT contraction differed by <5% when compared with the one preceding ODQ application. Inhibition of sGC with ODQ (Figure 2) resulted in a significant reduction of BAY 41-2272–evoked relaxations, as evidenced by the marked rightward shifts (≈10-fold) in both endothelium-intact (8.08 ± 0.04 in the absence and 7.11 ± 0.07 in the presence of ODQ; P < 0.01; n = 6) and denuded preparations (7.54 ± 0.05 in the absence and 6.59 ± 0.09 in the presence of ODQ; P < 0.01; n = 4). ODQ (n = 5) also virtually abolished maximal relaxations (10 μmol/L) elicited by ACh [control (CTL): 70 ± 5%; ODQ: 9 ± 1%] and GTN (CTL: 87 ± 3%; ODQ: 5 ± 1%).

Effect of NO Synthesis Inhibitors

In endothelium-intact rings pretreated with L-NAME (100 μmol/L), BAY 41-2272–induced relaxations were significantly attenuated (Figure 3; n = 5). The pEC50 values were 8.13 ± 0.02 in the absence and 7.53 ± 0.04 in the presence of L-NAME (P < 0.01). Similar to ODQ, L-NAME increased the basal tone by 36 ± 7% in intact rings. Again, the steady state of 5-HT contraction differed by <5% when compared with the one preceding L-NAME application; therefore, it was not necessary to correct for this effect. Unexpectedly, L-NAME also shifted the curves to BAY 41-2272 to the right in endothelium-denuded vessels (7.59 ± 0.07 in the absence and 7.13 ± 0.03 in the presence of L-NAME; P < 0.01; n = 4). To additionally investigate the role of NO in the denuded preparations, we tested the effects of selective nNOS inhibitors on BAY 41-2272 responses. Figure 3 shows that preincubation with S-methyl-L-thiocitrulline (10 μmol/L; n = 5) or 3-Br-7-NI (100 μmol/L; n = 4) caused significant rightward shifts (P < 0.01) in the relaxant curves elicited by BAY 41-2272 (CTL: 7.68 ± 0.05, S-methyl-L-thiocitrulline: 7.18 ± 0.08; CTL: 7.79 ± 0.07, 3-Br-7-NI: 7.20 ± 0.09). L-NAME, S-methyl-L-thiocitrulline, and 3-Br-7-NI had no significant effect on the baseline force in denuded vessels (6 ± 4%; 5 ± 2%, and 3 ± 1%, respectively). The maximal relaxations evoked by ACh were markedly reduced by L-NAME (CTL: 73 ± 6%; L-NAME: 13 ± 2%; P < 0.01) but not significantly affected by S-methyl-L-thiocitrulline (CTL: 63 ± 4%; S-methyl-L-thiocitrulline: 56 ± 8%) or 3-Br-7-NI (CTL: 60 ± 7%; 3-Br-7-NI: 50 ± 5%).

Effects of Sildenafil and K+ Channel Blockers

The selective phosphodiesterase type 5 inhibitor sildenafil (0.1 μmol/L) to endothelium-intact rings significantly potentiated the vasorelaxations induced by BAY 41-2272 (7.96 ± 0.06 in the absence and 8.49 ± 0.04 in the presence of sildenafil; P < 0.01; n = 4). Sildenafil also enhanced the relaxant responses evoked by BAY 41-2272 in denuded preparations with an ≈-fold leftward shift. Similar results were obtained with GTN (7.24 ± 0.06 in the absence and 7.72 ± 0.06 in the presence of sildenafil; P < 0.05; n = 5). The relaxant responses to BAY 41-2272 were unchanged in endothelium-denuded rings treated with selective blockers of ATP-dependent, voltage-dependent, and Ca2+-activated K+ channels of high and low conductance, respectively, glybenclamide (10 μmol/L; n = 5), 4-aminopyridine (1 mmol/L; n = 4), charybdotoxin (0.1 μmol/L; n = 6), and apamin (1 μmol/L; n = 4).

Effects of Ouabain

Pretreatment of denuded rings with 10 μmol/L ouabain (n = 5) had a significant inhibitory effect on the vasorelaxations elicited by BAY 41-2272 (7.64 ± 0.05 in the absence and 7.15 ± 0.05 in the presence of ouabain; P < 0.01). To examine whether Na+–K+–ATPase stimulation results from a direct effect or from cGMP formation in response to BAY 41-2272, we investigated the effects of ODQ and ouabain on BAY 41-2272–induced relaxations. The inhibition of BAY 41-2272 responses because of this combination resulted in a significant 10-fold rightward shift (CTL: 7.74 ± 0.04; ODQ + ouabain: 6.76 ± 0.09; P < 0.01), although not statistically different from the effects of ODQ alone.

Contractile Responses to CaCl2

Cumulative addition of CaCl2 (0.01 to 10 mmol/L) in the presence of K+-depolarized endothelium-denuded rings was used to evaluate contractions dependent on Ca2+ influx. Pretreatment with the L-type Ca2+ channel blocker nifedipine (1 μmol/L; n = 4) fully blocked contractile responses to CaCl2 (Figure 4; P < 0.01). In the presence of ODQ, BAY 41-2272 at 1 μmol/L, but not 0.1 μmol/L, considerably depressed maximal contractions to CaCl2 (35 ± 3% in the absence and
17±1% in the presence of BAY 41-2272; \( P<0.01; n=4 \) and caused an \( \approx 2.5 \)-fold rightward shift in the curves (Figure 4).

**Contractile Responses to 5-HT**

Cumulative addition of 5-HT (0.001 to 10 \( \mu \)mol/L) caused concentration-dependent contractions in denuded vessels with \( \text{pEC}_{50} \) and maximum response values averaging 7.09±0.03 and 121±3%, respectively. When applied alone, BAY 41-2272 (0.0001 to 0.1 \( \mu \)mol/L) caused concentration-dependent rightward shifts, whereas GTN (0.0001 to 0.1 \( \mu \)mol/L) caused a significant 2.5-fold shift only at the maximum concentration used (Figure 5; \( n=5 \), each). Then, concentrations that did not cause significant shifts were selected. Figure 5 shows that the coincubation of BAY 41-2272 (0.01 \( \mu \)mol/L) and GTN (0.01 \( \mu \)mol/L) resulted in marked rightward shifts of \( \approx 6 \)-fold (\( n=7 \)) versus the 1.7-fold (BAY 41-2272) and 1.2-fold (GTN) displacement when assayed separately (\( P<0.01 \)). A synergistic interaction between BAY 41-2272 and GTN was also obtained when measuring the percentage of inhibition of maximal 5-HT–induced contractions (Figure 5).

**Cyclic Nucleotide Contents**

Brain stem samples treated with BAY 41-2272 (0.1 \( \mu \)mol/L) or GTN (10 \( \mu \)mol/L) significantly increased cGMP levels by \( \approx 10 \)-fold and 4-fold, respectively (\( P<0.01; n=4 \)) in an ODQ (10 \( \mu \)mol/L)-sensitive manner. The coincubation of these drugs resulted in a marked synergistic increase in cGMP concentration of \( \approx 50 \)-fold over baseline levels (\( P<0.01; n=4 \)). Forskolin (1 \( \mu \)mol/L; \( n=4 \)) significantly increased cAMP but not cGMP levels (\( P<0.01 \)). Neither BAY 41-2272 nor GTN affected baseline cAMP readings at the concentrations used (Table).

**Discussion**

We report a detailed pharmacological analysis of the mechanisms involved in the vasorelaxation of rat basilar artery elicited by BAY 41-2272. There are 2 major findings in the present study. First, BAY 41-2272 exerts its relaxant effects through both cGMP-dependent and -independent mechanisms. Second, BAY 41-2272 relaxes the basilar artery in a synergistic fashion with endogenous and exogenous NO, both at functional and molecular levels, confirming the original hypothesis. The findings herein provide additional insight into the functional importance of sGC in cerebral arteries.

mRNA and protein for sGC have been demonstrated previously in cerebral blood vessels.\(^{21,22}\) Direct NO-independent stimulators of sGC have been developed recently based on the derivative YC-1.\(^{19}\) The cysteine 238 and cysteine 243 spanning region in the \( \alpha \)-subunit of the enzyme
Figure 5. Rightward shifts of the curves elicited by 5-HT (0.001 to 10 μmol/L) a) and percentage inhibition of maximal responses (Emax; b) in the presence of BAY 41-2272 (0.001 to 1 μmol/L; □) or glibenclamide trihydrate (GTN, 0.001 to 1 μmol/L; □) in endothelium-denuded rings (n=5). Rightward shifts (□) and percentage inhibition of Emax (■) in presence of BAY 41-2272 (0.01 μmol/L) and GTN (0.01 μmol/L) alone or in combination (c; n=7). Data represent the mean±SEM of n experiments.

was identified as part of the target site for BAY 41-2272, in addition to the heme group.19 Our results clearly showed that BAY 41-2272 concentration-dependently relaxed precontracted rings of basilar artery and significantly increased cGMP levels in brain stem slices in an ODQ-sensitive manner, consistent with the capacity to directly activate sGC. Indeed, previous studies using ODQ have demonstrated a profound inhibition of cGMP formation and NO-mediated relaxation of cerebral arteries in vitro and in vivo, consistent with the concept that cerebral vascular effects of NO result from activation of sGC and generation of cGMP.9,23,24 The findings that BAY 41-2272 produced ODQ-sensitive vasorelaxation corroborates the results from rabbit aorta20 and the heme dependency to stimulate sGC.19 The observation that ODQ virtually abolished cGMP increases elicited by BAY 41-2272 is in agreement with the inability of BAY 41-2272 to stimulate the recombinant enzyme in the presence of ODQ.19 Furthermore, the findings that the cGMP-specific phosphodiesterase type 5 inhibitor sildenafil enhanced BAY 41-2272 relaxations, as well as the demonstration that BAY 41-2272 increases cGMP, but not cAMP levels, confirms the key role of cGMP in these responses.

Our results demonstrate that BAY 41-2272 responses were attenuated in endothelium-denuded basilar artery, indicating that this response is partially dependent on endothelium integrity. Recently, BAY 41-2272–induced relaxations have been shown to be composed of an endothelium-dependent component in the rabbit aorta,20 supporting our observations. The findings that the broad spectrum NOS inhibitor L-NAME significantly reduced BAY 41-2272–evoked vasorelaxations in intact basilar artery rings suggest the requirement of endogenous NO in these responses. Accordingly, similar results were reported in rabbit aorta20 and corpus cavernosum.25

An intriguing observation was the demonstration that L-NAME also attenuates BAY 41-2272 responses in the absence of a functional endothelium. NOS immunoreactivity has been demonstrated previously in cerebrovascular nerve fibers and endothelial cells, indicating the sources of NO in cerebral blood vessels.26,27 The release of NO from perivascular nerves is one of the important neuroeffector mechanisms in the regulation of vasodilatory responses of cerebral arteries.28,29 Because selective nNOS inhibitors, such as S-methyl-L-thiocitrulline and 3-Br-7-NI, antagonized BAY 41-2272 responses in denuded vessels, the involvement of endogenously released NO in the vasorelaxant activity was additionally confirmed. The observation that L-NAME fails to alter the resting tone of denuded rings suggests that nNOS is not involved in the basal NO production. Instead, endothelium-derived NO release appears to be responsible for the maintenance of the resting dilator tone in the rat basilar artery. However, in vivo inhibition of nNOS reduces cerebral blood flow in rats, suggesting that NO released from neurons and/or glial cells may dilate intraparenchymal vessels to maintain the resting cerebral blood flow.30–32 It is unclear whether BAY 41-2272 releases NO from endothelial/neural sources or synergizes with endogenously generated NO to evoke vasorelaxation, although the BAY 41-2272 precursor YC-1 has been shown to stimulate NO production through
activation of endothelial NOS in bovine endothelial cells. In assays using purified sGC, BAY 41-2272 and NO donors synergize over a wide range of concentrations to stimulate enzyme activity. Similarly, we have successfully demonstrated a synergistic interaction between BAY 41-2272 and GTN to increase cGMP in brain stem slices. Functionally, this synergistic interaction caused a large decrease in both 5-HT sensitivity and efficacy to contract basilar artery rings, likely because of a marked increase in cGMP levels.

In some cerebral blood vessels, NO relaxation appears to involve cGMP-dependent activation of K⁺ channels. Nevertheless, blockers of the different K⁺ channel subtypes (ATP-dependent, voltage-dependent, and Ca²⁺-activated K⁺ channels of high and low conductance) did not change the effects of BAY 41-2272, arguing against the involvement of membrane hyperpolarization through K⁺ channel opening in the relaxations induced by this compound. Sarcolemmal Na⁺-K⁺-ATPase plays an important role in the regulation of vascular smooth muscle tone. An increase in pump activity induces vasorelaxation through stimulation of Na⁺/Ca²⁺ exchange and a reduction in Ca²⁺ influx via voltage-dependent Ca²⁺ channels. Although BAY 41-2272 has been shown recently to stimulate the Na⁺-K⁺-ATPase in a cGMP-independent manner in ovine pulmonary artery, it is evident from the present study that the relaxant effect in the basilar artery involves cGMP-mediated stimulation of the Na⁺-K⁺-ATPase pump, because the inhibitory effects of ouabain and ODQ were not additive. A rise in intracellular cGMP concentration, as well as activation of Na⁺-K⁺-ATPase, may affect Ca²⁺ movement/sensitivity of the contractile apparatus to Ca²⁺. This is evident from our results, because inhibition of Ca²⁺ influx clearly accounts for part of the relaxant mechanism of this drug. However, it is noteworthy that BAY 41-2272 antagonized Ca²⁺ influx in a cGMP-independent manner. Accordingly, BAY 41-2272 and YC-1 have been shown to inhibit Ca²⁺ entry by mechanisms that do not involve cGMP. In conclusion, the present study demonstrated that BAY 41-2272 causes basilar artery relaxation in a synergistic fashion with endogenous or exogenous NO. Our results demonstrate that, besides stimulating sGC, inhibition of Ca²⁺ entry also represents an important mechanism in BAY 41-2272-induced relaxation of the rat basilar artery. This dual mechanism suggests that NO-independent sGC stimulators are of particular clinical interest in the management of cerebrovascular disorders, particularly in situations where NO production is decreased.

Perspectives
Disruption of the NO-cGMP vasodilator mechanism may contribute to many of the functional changes observed in the development of chronic vasospasm after subarachnoid hemorrhage. NO donors or NOS substrates ameliorate cerebral vasospasm in both experimental and clinical settings. However, the short half-life, unpredictable pharmacokinetics, and potential toxicity limit the clinical use of NO for the treatment of cerebral vasospasm. Therefore, NO-independent sGC stimulators like BAY 41-2272 may offer efficacy and safety advantages over NO-based therapies, thus representing a promising therapeutic intervention in the management of chronic vasospasm.

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References


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