Inhibition of Phosphodiesterase Type 5 by the Activator of Nitric Oxide–Sensitive Guanylyl Cyclase BAY 41-2272

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Background—By the formation of cGMP, nitric oxide (NO)–sensitive guanylyl cyclase (GC) acts as the effector for the signaling molecule NO and mediates the relaxation of vascular smooth muscle and the inhibition of platelet aggregation. The compounds YC-1 and BAY 41-2272 are regarded as NO-independent activators and sensitizers of NO-sensitive GC. In vivo effects, for example, lowering blood pressure and prolonging tail-bleeding times, turn the compounds into promising candidates for the therapy of cardiovascular diseases. However, YC-1 has also been shown to inhibit the major cGMP-degrading enzyme phosphodiesterase type 5 (PDE5). The synergistic properties of YC-1 on cGMP formation and degradation lead to an excessive NO-induced cGMP accumulation in cells, explaining the observed physiological effects. We assessed a potential inhibition of PDE5 by the new GC activator BAY 41-2272.

Methods and Results—The effects of BAY 41-2272 on NO-sensitive GC and PDE5 activities were tested in vitro. BAY 41-2272 not only sensitized NO-sensitive GC toward activation by NO but also, with comparable potency, inhibited cGMP degradation by PDE5. In intact platelets, BAY 41-2272 greatly potentiated the NO-induced cGMP response that was caused by a synergistic effect of BAY 41-2272 on cGMP formation and degradation.

Conclusions—The physiological effects of BAY 41-2272, which are commonly ascribed to the NO-independent activation of NO-sensitive GC, are rather due to the synergism of sensitization of NO-sensitive GC and inhibition of PDE5. (Circulation. 2004;109:1711-1713.)

Key Words: inhibitors ■ nitric oxide ■ pharmacology ■ platelets

Nitric oxide (NO)–sensitive guanylyl cyclase (GC) catalyzes the formation of the intracellular messenger cGMP and is generally accepted as the most important receptor for the signaling molecule NO. The NO/cGMP pathway plays a role in a variety of physiological processes such as the relaxation of smooth muscle and the inhibition of platelet aggregation. A cGMP signal is terminated by the action of phosphodiesterases that hydrolyze cGMP. In many cell types such as vascular smooth muscle cells and platelets, the cGMP-specific phosphodiesterase type 5 (PDE5) is a major cGMP-degrading enzyme.1,2

NO-releasing substances, for example, glyceryl trinitrate, are widely used in the therapy of coronary heart disease. In vivo, the relaxation of venous smooth muscle is the primary action of these compounds. The resulting decrease in cardiac preload reduces ventricular wall tension and myocardial oxygen demand. Drawbacks in the use of organic nitrates are the development of tolerance and their poor antiplatelet properties. The phenomenon of tolerance to nitrates is not completely understood; various factors, for example, reduced bioconversion to NO, enhanced NO consumption by superoxide anions, and mechanisms acting downstream of NO, that is, on the level of NO-sensitive guanylyl cyclase and/or cGMP-degrading PDEs, have been proposed.3 In recent years, major efforts have been made to develop non-NO activators of guanylyl cyclase. YC-1 was the first substance discovered that displayed antplatelet properties by increasing intracellular cGMP through stimulation of NO-sensitive GC.4,5 Thorough investigations of the mechanism of action then revealed that the sensitization of NO-sensitive GC toward activation by NO and CO was the more striking feature of YC-1.6 Recently, the discovery of BAY 41-2272, a more potent sensitizer of NO-sensitive GC, has been reported.7 In vivo experiments with rats revealed a desirable hemodynamic effect of the compounds, for example, lowering of blood pressure.7,8 Furthermore, BAY 41-2272–treated animals did not develop tolerance. In sum, BAY 41-2272 and related substances acting as NO sensitizers are pharmacological agents of high potential for the treatment of cardiovascular disorders.

However, the prominent increase in intraplatelet cGMP after NO stimulation in the presence of YC-1 was shown to be due to a dual action of YC-1, that is, stimulation of NO-sensitive GC and inhibition of PDE5.9 Thus, the observed hemodynamic and antiplatelet effects of YC-1 can be at least partially attributed to the inhibition of cGMP degradation. The in vivo effects of BAY 41-2272 have been solely attributed to its stimulatory effect on NO-sensitive GC, and the compound is widely used as an NO-independent activator.
of GC. In the original publication, an inhibitory effect of BAY 41-2272 on PDE5 was not observed up to a concentration of 10 μmol/L. In contrast, we demonstrated that BAY 41-2272 does inhibit PDE5, in addition to sensitization of GC toward NO. Thus, the actions of BAY 41-2272 on NO/cGMP signaling are similar to those of YC-1. Any physiological effects of BAY 41-2272 are therefore potentially caused by a combined action on NO-sensitive GC and PDE5 and must be interpreted with caution in the future.

**Methods**

Guanlyl cyclase assays were performed with the use of NO-sensitive GC, purified as described elsewhere. Human PDE5A1 cDNA (cloned by standard procedures from human placenta) was inserted into the pcDNA3 vector (Invitrogen), bearing the resistance against zeocin. Transfection into HEK293 cells was performed with the use of FuGENE 6 transfection reagent (Roche); individual clones of stably transfected cells were selected with the use of zeocin (200 μg/mL). Cells were grown to confluency, harvested (120,000 g), and lysed in 800 μL homogenization buffer (50 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L triethanolamine/HCl, pH 7.4, 2 mmol/L DTT, and a 100-fold dilution of protease inhibitor cocktail (Sigma Aldrich) by sonication (1 pulse, 5 seconds). Lysates were centrifuged (30 minutes, 50,000 g), and cytosolic fractions were used for PDE5 assays carried out as described. Isolation of washed human platelets and radioimmunoassay for cGMP was performed as reported. S-nitroso-glutathione (GSNO), DEA-NO, YC-1, and BAY 41-2272 were purchased from Alexis; erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was from Tocris Cookson. Sildenafil was kindly supplied by Pfizer.

**Results**

The effect of BAY 41-2272 on purified NO-sensitive GC was studied first to reassess its mechanism of action on our enzyme preparation. Activity of NO-sensitive GC was determined at increasing concentrations of the NO donor DEA-NO in the absence or presence of BAY 41-2272. As can be seen in Figure 1A, the presence of BAY 41-2272, the concentration response for DEA-NO was shifted to the left by ∼1.5 orders of magnitude; the EC₅₀ values for DEA-NO were 630 nmol/L and 17 nmol/L in the absence or presence of BAY 41-2272, respectively. The data establish that BAY 41-2272 sensitizes NO-sensitive GC for stimulation by NO even more effectively than YC-1, which shifts the concentration response by 1 order of magnitude. BAY 41-2272 alone caused an ∼30-fold activation of NO-sensitive GC. The maximal NO-stimulated activity was increased by ∼20% in the presence of BAY 41-2272. The results clearly suggest a mechanism of action similar to the one reported for YC-1, that is, sensitization of NO-sensitive GC by inhibition of deactivation, most likely by inhibition of NO dissociation from the prosthetic haem group of the enzyme. The EC₅₀ value for BAY 41-2272 activation as determined in the presence and absence of 100 nmol/L DEA-NO was 0.3 μmol/L and 3 μmol/L, respectively (Figure 1B).

The substance YC-1 has been shown to sensitize NO-sensitive GC and to also inhibit PDE5, which is the major enzyme responsible for cGMP degradation in many cell types. Therefore, we directly compared the effects of BAY 41-2272 and YC-1 on PDE5 catalytic activity. PDE5 activity was measured in the supernatant fraction of stably transfected HEK293 cells. Figure 1C shows that BAY 41-2272 indeed inhibited PDE5, with a slightly higher potency than YC-1; PDE5 activity was inhibited by 50% at 3 μmol/L BAY-2272 or 10 μmol/L YC-1 (at 0.1 μmol/L cGMP as substrate), respectively. The concentration-response curves for inhibition were shifted to the right at a higher substrate concentration. This indicates a competitive component in the mechanism of inhibition. The data imply that the hemodynamic and antiplatelet effects of BAY 41-2272 observed in vivo are caused by potentiation of cGMP accumulation resulting from the combination of GC sensitization and PDE5 inhibition.

In intact cells, cGMP levels always reflect the balance of cGMP formation by GCs and degradation by PDEs. The activation of NO-sensitive GC with a concomitant inhibition of cGMP degradation should thus lead to a potentiation of cGMP accumulation. Accordingly, the effect of BAY 41-
2272 on the NO-induced cGMP response was measured in human platelets and compared with the effects of YC-1 and the combination of PDE2 and PDE5 inhibitors (sildenafil and EHNA), respectively. Shown in Figure 2A are the cGMP responses elicited by a maximally effective NO concentration (100 μmol/L GSNO). The cGMP response induced by NO alone displayed the characteristic transient elevation of intracellular cGMP, with the decline of cGMP reflecting activation of PDE5.1,2 Accordingly, in the presence of PDE inhibitors, cGMP accumulated to a plateau of ≈3000 pmol cGMP/10⁹ platelets within a few seconds. In the presence of YC-1, a similar plateau was reached with comparable kinetics. The cGMP response in the presence of BAY 41-2272 reached slightly lower levels of 2000 pmol cGMP/10⁹ platelets. The tremendous NO-induced accumulation of cGMP in the presence of YC-1 or BAY-2272 can be solely explained by the inhibition of PDE5. The finding that the effect of BAY 41-2272 was somewhat less pronounced is consistent with the inhibition of PDE5 phosphorylation in smooth muscle cells.

To assess a possible sensitizing effect of BAY 41-2272 and YC-1 on NO-sensitive GC in platelets, a submaximally effective concentration of NO (3 μmol/L GSNO) was used. The cGMP response induced by this NO concentration in the absence of any compound was only marginal (Figure 2B). PDE inhibitors were used under the same conditions to unveil the activity of NO-sensitive GC; here, a linear cGMP accumulation was observed over a period of 60 seconds, which can be explained by subtle activation of NO-sensitive GC. On the other hand, the cGMP responses induced by 3 μmol/L GSNO in the presence of BAY 41-2272 or YC-1 (100 μmol/L, respectively) led to rapid increases in cGMP up to 1000 or 2000 pmol/10⁹ platelets, respectively (Figure 2B). Compared with the NO-induced cGMP accumulation in the presence of PDE inhibitors, the NO-induced cGMP formation was clearly potentiated by BAY-2272 and YC-1, demonstrating sensitization of NO-sensitive GC in intact cells.

Discussion

In contrast to the results presented in a previous report,7 we demonstrate that BAY 41-2272 does inhibit PDE5. The concentrations required for PDE5 inhibition were in a range comparable to those required for GC sensitization. Accordingly, the physiological effects of the compound observed in intact cells or in animal models must be attributed to a synergistic effect on cGMP formation and degradation by sensitization of NO-sensitive GC and inhibition of PDE5. Thus, the lack of tolerance observed in BAY 41-2272–treated animals is not surprising and may well be a result of PDE5 inhibition. Considering the occurrence of physiological concentrations of NO, the combination of sensitization of NO-sensitive GC and PDE5 inhibition render substances such as YC-1 and BAY 41-2272 powerful cGMP-elevating agents. On the other hand, the potentiation of cGMP accumulation may also cause considerable side effects such as severe hypotension, known from the simultaneous application of sildenafil and NO donors.

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References

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