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YC-1-Like Potentiation of NO-Dependent Activation of Soluble Guanylate Cyclase by Derivatives of Protoporphyrin IX

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Abstract—The influence of protoporphyrin IX derivatives—2,4-di(1-methoxyethyl)-deuteroporphyrin IX disodium salt (dimegin) and hematoporphyrin IX (HP)—on the activation of human platelet soluble guanylate cyclase by sodium nitroprusside was investigated. Dimegin and HP, like 1-benzyl-3-(hydroxymethyl-2-furyl)indazole (YC-1), produce synergistic effects on the activation of soluble guanylate cyclase by sodium nitroprusside. The synergistic activation of the enzyme by the combination of 10 μM sodium nitroprusside and 5 μM dimegin (or 5 μM HP) was 190 ± 19 and 134 ± 10%, respectively. The synergistic activation of guanylate cyclase by 3 μM YC-1 and 10 μM sodium nitroprusside was 255 ± 19%. Dimegin and HP had no effect on the activation of guanylate cyclase by YC-1; they did not change the synergistic effect of YC-1 (3 μM) and sodium nitroprusside (10 μM) on guanylate cyclase activity. The synergistic activation of NO-stimulated guanylate cyclase activity by dimegin and HP represents a new biochemical effect of these compounds that may have important pharmacotherapeutic and physiological significance.

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The soluble heme-containing guanylate cyclase is the main intracellular receptor for nitric oxide (NO). NO activates the enzyme and facilitates the formation of the second messenger cyclic guanosine-3′,5′-monophosphate (cGMP). The latter mediates a wide range of physiological functions via interaction with specific cGMP-dependent protein kinase, ion channels, and phosphodiesterase [1, 2]. This signal transduction pathway underlies the majority of physiological actions attributed to NO; it is important in the regulation of the cardiovascular, gastrointestinal, and immune systems. The consequence of action of the signaling NO–soluble guanylate cyclase–cGMP system is important for the etiology of various pathological states.

Agents that can selectively modulate enzyme activity may possess considerable therapeutic potential [2]. It is known that endogenous NO formed from L-arginine under the action of L-arginine-NO-synthase [3], which is identical to endothelium derived relaxation factor (EDRF) [4], appears to be a neurotransmitter [5], cytotoxic agent [6], and powerful factor of hemostasis. It inhibits platelets aggregation [7] and is presently considered as an endogenous vasodilator. The vasodilatory action of NO is connected with the activation of soluble guanylate cyclase as a result of interaction with the guanylate cyclase heme and formation of the nitrosyl–heme complex [8]. Under these conditions, the iron protrudes the plane of the porphyrin ring and the structure of the nitrosyl–heme complex formed (the real activator of the enzyme) acquires resemblance to the structure of protoporphyrin IX (PTP)—one of the most potent activators of guanylate cyclase.

The use of organic nitrates (e.g., glyceryl trinitrate) for the treatment of conditions such as angina and heart failure has been advocated for over a century [9]. Organic nitrates are widely used at the present time as well. However, the mechanism of action of such compounds (and other NO-donors) was not elucidated until the late 1970s. Subsequently, it was found to involve the metabolic conversion to NO, which was similar to the mechanism of vasodilatory action of endogenous nitric oxide. At present, a great number of guanylate cyclase activators

Abbreviations: PTP) protoporphyrin IX; dimegin) 2,4-di(1-methoxyethyl)-deuteroporphyrin IX disodium salt; HP) hematoporphyrin IX; YC-1) 1-benzyl-3-(5′-hydroxymethyl-2′-furyl)indazole.

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relating to NO-donors are known. However, the search for new analogous compounds still goes on, but the use of such compounds raises several problems [1]. First, NO-donor compounds, particularly organic nitrates, induce the development of tolerance following prolonged administration [10]. The mechanism(s) underlying this tolerance remain unclear, but may be linked to decreased metabolic activation of the compounds [10], excessive superoxide, endothelin or angiotensin II levels [11], or reduction in the sensitivity/activity of NO receptor—soluble guanylate cyclase [12]. Second, the use of NO donors *in vivo* is potentially troublesome due to nonspecific interaction of NO with other biological molecules; reactions that are difficult to control due to spontaneous release of NO from nitrovasodilators and its free diffusion in biological systems. In light of these shortcomings, compounds that can activate soluble guanylate cyclase in an NO-independent manner, and not cause tolerance, will therefore offer considerable advance on current therapy.

Stasch et al. [13-16] have reported such a series of compounds, derivatives of pyrazolopyridine: BAY 41-2272, BAY 41-8543, and BAY 51-9491. They have studied the mechanism of guanylate cyclase activation by these compounds and their pharmacological properties.

This family of compounds is based on YC-1 (1-benzyl-3-(5′-hydroxymethyl-2′-furyl)indazole) [17]. YC-1 not only activates soluble guanylate cyclase in an NO-independent mechanism but increases the activation of the enzyme by NO donors as well. In the presence of both YC-1 and an NO donor, there is a concentration-dependent (additive or synergistic) activation of guanylate cyclase [18, 19]. The ability of YC-1 to increase the efficiency of actions of endogenous NO (and NO donors) may have great pharmacotherapeutic and physiological significance. The use of compounds possessing YC-1 like action will allow reducing the doses of nitrovasodilators (and other NO donors) and therefore, to diminish or to remove undesirable site effects without lowering the efficiency of their therapeutic action [20].

In this study, we have investigated derivatives of PTP containing at positions 2 and 4 of the macrocycle: hydroxyethyl (hematoporphyrin IX, HP) and methoxyethyl (dimegin) substitutes, respectively, instead of vinyl groups. These compounds not only activated human platelet soluble guanylate cyclase but synergistically increased the stimulation of the enzyme activity by NO donor (sodium nitroprusside).

MATERIALS AND METHODS

Human platelets were used as a source of soluble guanylate cyclase. Platelets were isolated from the blood of healthy donors as described earlier [21]. A suspension of washed platelets in 50 mM Tris-HCl buffer (pH 7.6) containing 0.2 mM dithiothreitol was sonicated in an MSE 5-78 ultrasonic disintegrator (UK) for 20 sec at 2°C and centrifuged at 105,000*g* for 1 h. The supernatant was used as a source of human platelet soluble guanylate cyclase.

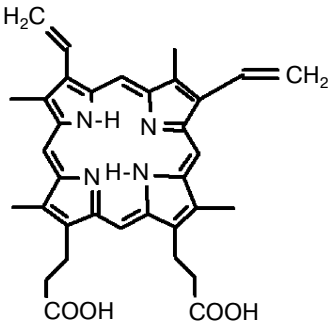
Guanylate cyclase activity was assayed as described [22]. Briefly, the samples (final volume 150 μl) contained 50 mM Tris-HCl buffer (pH 7.6), 1 mM GTP, 4 μM $MgCl_2$, 4 μM creatine phosphate, 20 μg (120-160 units) creatine phosphokinase, 10 μM theophylline, 20 μg of human platelet 105,000*g* supernatant, and other additives. The PTP derivatives (dimegin and HP) were used in the concentration range 0.1-10 μM. To compare the effect of dimegin, PTP, and HP on the basal activity of soluble guanylate cyclase and activation of the enzyme by sodium nitroprusside, 5 μM concentrations of the compounds were used. The compounds were first preincubated (10 min at 2°C) with guanylate cyclase before the addition of NO donor. Because of the poor solubility of PTP derivatives used in the buffer solution, they were initially dissolved in dimethyl sulfoxide (DMSO) with subsequent dilution in 50 mM Tris-HCl buffer (pH 7.6) to the required concentration. Control samples contained the same amount of DMSO.

The amount of cGMP formed (15 min, 37°C) was estimated by an enzyme-linked immunosorbent assay (ELISA) method using kits for quantitative determination of cGMP produced in Russia. Protein was determined by the Bradford method [23].

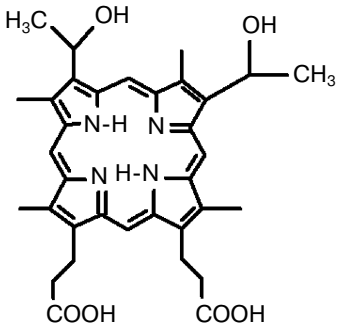
The following reagents were used: GTP sodium salt (Fluka, Switzerland); PTP and HP (Aldrich, UK); dimegin was synthesized from hematoporphyrin, tetramethyl ether [24], according to [25]. Other reagents were from Sigma (USA).

RESULTS

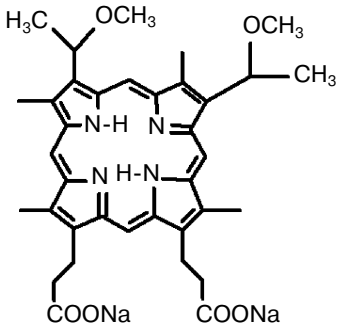
Chemical structures of the synthesized protoporphyrin IX (PTP) derivatives, dimegin and hematoporphyrin IX (HP), are given below. Dimegin and HP stimulate human platelet soluble guanylate cyclase activity. The basal enzyme activity was 91 ± 6 pmol cGMP/min per mg protein without compounds and 118 ± 8, 164 ± 12, 291 ± 21, and 273 ± 19 pmol cGMP/min per mg protein in the presence of 0.1, 1, 5, and 10 μM dimegin and 127 ± 7, 182 ± 12, 246 ± 18, and 205 ± 15 pmol cGMP/min per mg protein in the presence of the same concentrations of HP, respectively; i.e., at the optimal (for both compounds) 5 μM concentration of dimegin or HP, the degree of guanylate cyclase activation was 3.2- and 2.7-fold, respectively. Earlier it was shown that the optimal concentration for the activation of human platelet guanylate cyclase by PTP was 5 μM also (data not shown). In the present experiments the basal enzyme activity in the presence of 5 μM PTP was 321 ± 24 pmol



PTP



HP



Dimegin

cGMP/min per mg protein, i.e., the degree of activation was 3.5-fold. In other words, the efficiency of the stimulatory effects of PTP and its derivatives, dimegin and HP, was rather close.

Pilot experiments revealed that dimegin and HP potentiate the sodium nitroprusside-induced activation of soluble guanylate cyclase in a concentration-dependent manner. The maximal stimulation was observed at 5 μM concentrations of these compounds (data not shown). Table 1 demonstrates that increase in guanylate cyclase activation in the presence of both 10 μM sodium nitroprusside and 5 μM dimegin (or 5 μM HP) is 190 ± 19% (or 134 ± 10%). This is indicating synergistic activation of the enzyme by NO donor. The synergistic increase in guanylate cyclase activation by 10 μM sodium nitroprusside also occurred in the presence of 3 μM YC-1 (255 ± 19%, Table 1). It should be noted that dimegin and HP had no influence on the activation of the enzyme by YC-1. Table 1 shows that the activity of guanylate cyclase incubated with 3 μM YC-1 and 5 μM dimegin (or 5 μM HP) together was very close to the arithmetic sum of the enzyme activities stimulated by each agent alone. This means that dimegin and HP act on the enzyme independently from YC-1.

Unlike the synergistic effect of YC-1 on sodium nitroprusside-induced activation of guanylate cyclase, the effects of YC-1 (3 μM) and PTP (5 μM) incubated with the enzyme together were additive (see Table 2). The same additive effect was found during the incubation of the enzyme in the presence of both 10 μM sodium nitroprusside and 5 μM PTP (Table 2). At the same time, the activation of soluble guanylate cyclase by PTP in the presence of 5 μM dimegin (or 5 μM HP) was inhibited by 33 ± 2 (or 30 ± 2%).

Thus, dimegin (5 μM) and HP (5 μM) provoke a synergistic effect on sodium nitroprusside (10 μM)-

Table 1. Effect of dimegin (5 μM), hematoporphyrin IX (HP) (5 μM), and YC-1 (3 μM) on the activation of human platelet soluble guanylate cyclase (sGC) by sodium nitroprusside (SNP) (10 μM)

Compounds and additives	Stimulated activity of sGC, pmol cGMP/min per mg protein	Arithmetic sum of sGC activities alone	Effect (%) of:		
			YC-1	dimegin	HP
SNP	422 ± 30				
YC-1	42 ± 3				
YC-1 + SNP	1121 ± 89	464 ± 37	+255 ± 19		
Dimegin	154 ± 9				
Dimegin + SNP	1099 ± 88	576 ± 46		+190 ± 19	
Dimegin + YC-1	191 ± 13	196 ± 13		+7	
Dimegin + SNP + YC-1	1655 ± 136	618 ± 49		+268 ± 22	
HP	117 ± 8				
HP + SNP	723 ± 51	539 ± 36			+134 ± 10
HP + YC-1	163 ± 11	159 ± 11			−3
HP + SNP + YC-1	1351 ± 115	581 ± 47			+232 ± 18

Note: Data represent mean ± S.D. of 4-5 independent experiments with <10% error. Basal guanylate cyclase activity was 76 ± 6 pmol cGMP/min per mg protein.

Table 2. Effect of dimegin (5 μM), hematoporphyrin IX (HP) (5 μM), sodium nitroprusside (SNP) (10 μM), and YC-1 (3 μM) on the activation of human platelet soluble guanylate cyclase (sGC) by protoporphyrin IX (PTP) (5 μM)

Compounds and additives	Stimulated activity of sGC, pmol cGMP/min per mg protein	Arithmetic sum of sGC activities alone	Effect (%) of:			
			YC-1	SNP	dimegin	HP
PTP	259 ± 18					
YC-1	149 ± 11					
YC-1 + PTP	437 ± 32	408 ± 29	107 ± 7			
SNP	360 ± 29					
SNP + PTP	600 ± 45	619 ± 46		97 ± 6		
Dimegin	200 ± 12					
Dimegin + PTP	308 ± 22	459 ± 33			−33 ± 2	
HP	177 ± 12					
HP + PTP	331 ± 24	475 ± 34				−30 ± 2

Note: Data represent mean ± S.D. of four independent experiments with <10% error. Basal guanylate cyclase activity was 70 ± 5 pmol cGMP/min per mg protein.

induced activation of soluble guanylate cyclase; they have no influence on the activation of the enzyme by YC-1 (3 μM) and do not change a synergistic increase of sodium nitroprusside (10 μM)-induced activation of soluble guanylate cyclase in the presence of YC-1 (3 μM).

DISCUSSION

The data presented here demonstrate for the first time that the derivatives of PTP used—dimegin and HP—are NO-independent activators of soluble guanylate cyclase and produce a synergistic effect on the activation of this enzyme by sodium nitroprusside (NO donor). Increase in basal guanylate cyclase activity with the increase in the concentrations of PTP derivatives used (from 0.1 to 10 μM) is consistent with literature data that some porphyrins may activate soluble guanylate cyclase [26]. The effectiveness of such activation depends on the structure of the porphyrin.

The most potent enzyme stimulator is PTP—a heme precursor. The hydrophobic vinyl side chains at positions 2 and 4 and vicinal propionic acid residues at positions 6 and 7 of the porphyrin ring are important structural elements of the PTP molecule [26]. Hydrophobic interactions between porphyrins and guanylate cyclase are essential for maximal enzyme activation. The lowering in hydrophobic properties of vicinal residues (for example, the substitution of the hydrogen atoms with hydroxyl groups) may weaken the activating effect of porphyrins [26]. However, in our experiments significant differences in guanylate cyclase activation by the compounds used were not observed. The stimulatory effects of dimegin, HP, and PTP were similar in spite of substitution of two hydrogen atoms in two vinyl side chains of the PTP molecule with two hydroxyl and two methoxyl groups in the HP and dimegin molecules, respectively. At the same time, these structural changes influenced the ability of the compounds to increase the activation of soluble guanylate cyclase by NO donors. It is known that YC-1 induces a synergistic effect on the activation of soluble guanylate cyclase by NO donors [16-19]. Dimegin and HP, but not PTP, possess the same property. The stimulatory effects of PTP (5 μM) and sodium nitroprusside (10 μM) incubated with the enzyme together were additive (Table 2). Activation of guanylate cyclase by a combination of sodium nitroprusside (10 μM) and dimegin (5 μM) or sodium nitroprusside (10 μM) and HP (5 μM) was synergistic and equal to 190 ± 19 and 134 ± 10%, respectively (Table 1). A synergistic activation of soluble guanylate cyclase by sodium nitroprusside (10 μM) in the presence of YC-1 (3 μM) was 255 ± 19% (Table 1).

The data presented here do not allow to conclude that dimegin (or HP) interacts with guanylate cyclase at the same binding sites as YC-1 [27, 28] because there was no competition between dimegin (or HP) and YC-1. Dimegin and HP have no influence on guanylate cyclase activation by YC-1 and do not change a synergistic effect of YC-1 on sodium nitroprusside-induced guanylate cyclase activation (Table 1). In all cases, dimegin and HP act independently from YC-1. YC-1 has no influence on PTP-induced guanylate cyclase activation. As in experiments with dimegin and HP, the stimulatory effects of PTP (5 μM) and YC-1 (3 μM) incubated with the enzyme together were additive (Table 2). However, dimegin and HP inhibit (by 32 ± 2 and 30 ± 2%, respectively, Table 2)

the PTP-induced guanylate cyclase activation indicating a competition between these compounds and PTP. This suggests that dimegin and HP interact (at least partially) with the enzyme at the same binding sites as PTP [26]. Dimegin and HP (like YC-1) induce a synergistic increase in guanylate cyclase activation by NO donors; PTP does not possess such ability. To explain this fact by the peculiarity of the chemical structures of these compounds seems not yet possible. Further investigations are necessary.

Thus, the data presented here on the ability of dimegin and HP to induce synergistic activation of guanylate cyclase by NO donors demonstrate for the first time a new biochemical effect of these compounds. This phenomenon may have great pharmacotherapeutic and physiological implications. Creation of compounds analogous to YC-1, which may increase the activation of guanylate cyclase by NO donors but devoid of the problem of tolerance, is important both as pharmacological tools and in development of new therapeutics.

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