The Role of Thiols in the Stimulation of Soluble Guanylate Cyclase by Compounds Generating Nitric Oxide

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The degree of guanylate cyclase activation by sodium nitroprusside increases in the presence of high concentrations of thiols, while its activation with diazetine-di-N-oxide derivatives remains unchanged. In contrast to conventional nitrovasodilators, these derivatives induce no tolerance resulting from deficiency or depletion of endogenous thiols.

Key Words: *guanylate cyclase; nitrogen oxide, nitrovasodilators*

Endogenous nitric oxide (NO) formed from L-arginine under the action of L-arginine-NO synthase [8] is a powerful hemostatic factor which is regarded as an endogenous vasodilator. Similarly to the vasodilatory activity of organic nitrates (nitroglycerin, etc.), its antihypertensive activity is associated with stimulation of soluble guanylate cyclase (GC; EC 4.6.1.2) and accumulation of cyclic 3',5'-guanosine monophosphate (cGMP). The mechanism of GC activation is as follows: NO or the NO group of nitrovasodilators binds to the GC heme with formation of the nitrosyl-heme complex, which is believed to be the true activator of GC [4]. Although organic nitrates have been widely used, they produce a number of undesirable side effects, the development of tolerance after a long-term therapy being the major one. Therefore, alternative vasodilators are necessary.

Since NO is generated from nitroglycerin and other organic nitrates as a result of their biotransformation, it can be concluded that vasodilatory activity of these compounds is mediated by a physiological mechanism. The findings that NO can be generated endogenously stimulated interest in nitrates [8]. Identification of new GC activators from which NO can be derived in the body opens new prospects in the search for new effective vasodilators.

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Diazetine-di-N-oxide derivatives are a novel class of compounds synthesized in Russia. They produce NO upon breakdown at physiological pH without enzymes. The compounds activate soluble GC, the effect being proportional to the amount of generated NO [9]. In addition, diazetine-di-N-oxide derivatives exhibit spasmolytics activity in pectoral muscle preparations and antihypertensive activity in spontaneously hypertensive rats anesthetized with Urethane [9]. In contrast to organic nitrates (nitroglycerin), diazetine-di-N-oxide derivatives generate NO in the absence of thiols [1]. The development of tolerance to nitrovasodilators has been linked to the deficiency or depletion of endogenous thiols.

In the present study we assessed the contribution of thiols to the activation of soluble GC by diazetine-di-N-oxide derivatives.

Generation of NO from these compounds is described by the following equation:

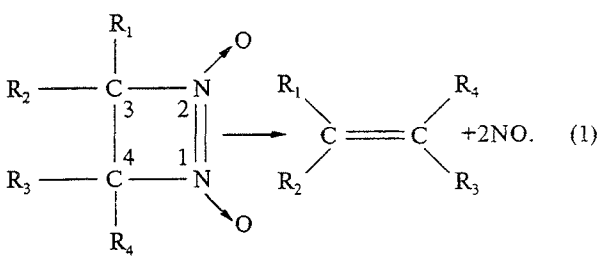

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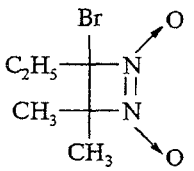
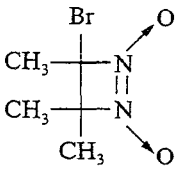
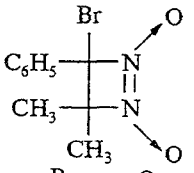
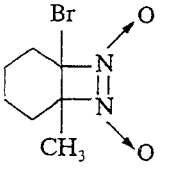
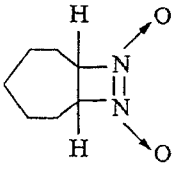
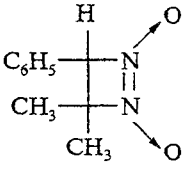
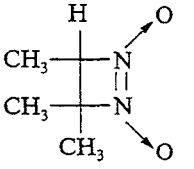
TABLE 1. Effects of 1,2-Diazetine-di-N-Oxides on the Activity of Soluble Guanylate Cyclase (GC) from Human Platelets

Compound No.	Name	Chemical formula	GC activation
1	5-Bromo-3-ethyl-4,4-dimethyl-1,2-diazetine-1,2-di-N-oxide		6.2±0.93
2	3-Bromo-3-methyl-4,4-dimethyl-1,2-diazetine-1,2-di-N-oxide		13.6±1.6
3	3-Bromo-3-phenyl-4,4-dimethyl-1,2-diazetine-1,2-di-N-oxide		9.1±0.83
4	3-Bromo-4-methyl-3,4-tetramethylene-1,2-diazetine-1,2-di-N-oxide		15.5±2.09
5	3,4-Pentamethylene-1,2-diazetine-1,2-di-N-oxide		2.6±0.15
6	3-Phenyl-4,4-dimethyl-1,2-diazetine-1,2-di-N-oxide		14.1±0.96
7	3-Methyl-4,4-dimethyl-1,2-diazetine-1,2-di-N-oxide		5.0±0.69
8	Sodium nitroprusside	$Na_2[Fe(CN)_5NO]$	7.3±0.56

Seven diazetine-di-N-oxide derivatives differing from each other by the substituents in positions 3 and 4 were analyzed for the ability to activate GC in comparison with sodium nitroprusside in the presence of varied thiol concentrations.

MATERIALS AND METHODS

The studied diazetine-di-N-oxide derivatives were synthesized by oxidation of α-hydroxylamines or 1,2-bis-hydroxylamines and were kindly supplied by Dr. L. B. Volodarskii [1,6].

Guanylate cyclase was isolated from human platelets. For this purpose washed platelets were suspended in 50 mM Tris-HCl buffer (pH 7.6) with and without 0.2 mM dithiothreitol (DTT), sonicated in an MSE 5-78 ultrasonic disintegrator for 20 sec at 0°C, and then centrifuged at 105,000*g* for 60 min. The supernatant was used as a preparation of soluble GC.

The enzyme activity was measured as described elsewhere [6]. Each sample (final volume 150 μl) contained 50 mM Tris-HCl buffer (pH 7.6), 1 mM GTP, 4 mM $MgCl_2$, 4 mM creatine phosphate, 20

μg (120-160 U) creatine phosphokinase, 10 mM theophylline, GC preparation (10-20 μg by protein), and other additives as appropriate. The diazetine-di-N-oxide derivatives and sodium nitroprusside were used in a concentration of 0.1 mM per sample. In the samples containing DTT, their final concentration was 2.7×10^{-5} or 3×10^{-4} M.

The amount of cGMP formed in the reaction (15 min at 37°C) was determined by ELISA using special kits (Meditsina. Analitika. Veterinariya, Russia). Protein content was determined by the method of Lowry [5].

The GTP sodium salt was from Fluka, sodium nitroprusside was from Chemapol, and other reagents were from Sigma.

RESULTS

Table 1 shows chemical structure of the studied diazetine-di-N-oxide derivatives. Their ability to activate soluble GC was compared with that of sodium nitroprusside. Compound 4 proved to be the strongest activator GC: its effect was twice as high as that of sodium nitroprusside. The activatory effects of compounds 2 and 6 were also higher than the effect of sodium nitroprusside. Compound 5 was the weakest activator of soluble GC. The effects of compounds 1, 3, and 7 on GC were virtually the same as that of sodium nitroprusside (Table 1). These results agree with our previous findings [9].

When platelet suspensions were sonicated in Tris-HCl buffer (pH 7.6) without DTT and DTT was not added to the reaction mixture, the specific activity of GC in the presence of compound 1-4, 6, or 7 was higher than that in the presence of sodium nitroprusside, while in the presence of compound 5 it was lower (Table 2). Taking as 100% the rise in the specific activity of GC from 150±10 (basal activity) to 413±24 pmol cGMP/min/mg protein (in the presence of sodium nitroprusside), compounds 1-4, 6, and 7 increased the enzyme activity to 139%, 247%, 158%, 220%, 249%, and 168%, respectively.

The addition of DTT to a final concentration of 2.7×10^{-5} M increased the specific activity of GC to 170% in the presence of sodium nitroprusside and had no effect on the enzyme activity in the presence of compounds 1-7 (Table 2).

An increase in the DTT concentration in the reaction mixture to 3.0×10^{-4} M led to a rise in the specific activity of GC to 270% in the presence of sodium nitroprusside but not in the presence of all the compounds except compound 4. However, the increase in the enzyme activity in the presence of compound 4 was statistically insignificant (Table 2).

Since thiols are necessary for NO generation from sodium nitroprusside, nitroglycerin, and other nitrovasodilators [7], the level of GC activation by sodium nitroprusside is higher at higher thiol concentrations in the reaction mixture [3] (equation 1). No thiols are required for NO generation from diazetine-di-N-oxide derivatives [1,6]; therefore, an increase in the DTT concentration had virtually no effect on GC activity in the presence of compounds 1-7.

Previously, we showed that the studied diazetine-di-N-oxide derivatives have spasmolytic and antihypotensive activities, the spasmolytic effect being comparable to that of nitroglycerin [9].

Since thiols are not required for NO generation from diazetine-di-N-oxide derivatives and for activation of soluble GC, these compounds are promising candidates for effective vasodilators with low side effects.

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TABLE 2. Effects of Dithiothreitol (DTT) on the Activity of Human Platelet Guanylate Cyclase (GC) in the Presence of Various Diazetine-di-N-Oxide Derivatives and Sodium Nitroprusside

Compound No.	Specific GC activity, pmol cGMP/min/mg protein		
	without DTT	with DTT, 2.7×10^{-5} M	with DTT, 3.0×10^{-4} M
1	573±75.6	630±93.2 (110)	642±91.8 (110)
2	1021±132.7	990±121.8 (97)	988±123.5 (97)
3	652±65.8	691±63.8 (106)	639±72.1 (98)
4	907±106.1	1172±158.0 (130)	1360±174.1 (150)
5	240±14.6	252±14.1 (105)	162±13.7 (70)
6	1030±59.7	1029±70.0 (100)	1082±66.0 (105)
7	693±43.6	485±66.9 (70)	554±100.2 (80)
Sodium nitroprusside	413±24.3	702±50.5 (170)	867±56.4 (210)

Note. An increase in specific GC activity (percent ratio of specific activity in the presence of DTT to that in its absence) is shown in parentheses. Basal specific activity in the samples without DTT (150±10.2 pmol cGMP/min/mg protein) was taken as 100%.

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Effect of the Leu-Enkephalin Analog Dalargin on DNA Synthesis in the Myocardium and Lingual Epithelium of Rats in Early Postnatal Ontogeny

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The DNA synthesis is studied in the myocardium and lingual epithelium of 4-5-day-old rats 24 h after a single administration of the leu-enkephalin analog dalargin in a dose of 10 or 100 μg/kg. At 10 μg/kg, dalargin significantly decreases the number of DNA-synthesizing nuclei in the left atrium, while at 100 μg/kg it significantly decreases the number of DNA-synthesizing nuclei in the left atrium and both ventricles. Dalargin does not change the DNA synthesis in the lingual epithelial cells.

Key Words: *dalargin; DNA synthesis; myocardium; epithelium; postnatal ontogeny*

Opioid peptides contribute to the maintenance of structural homeostasis in the body by regulating proliferation and differentiation processes [15]. The presence of endogenous opioid peptides in the mammalian myocardium [10] and the pronounced cardiotropic activity of exogenous opioids [5] suggest that opioids are involved in cardiac morphogenesis.

Our objective was to examine the effect of dalargin, a stable leu-enkephalin analog, on the DNA synthesis in rat myocardium during the early postnatal ontogeny.

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MATERIALS AND METHODS

Random-bred white rats aged 4-5 days were used. Control and experimental groups were formed by splitting litters so that there were 8 pups per nest. Experimental pups received 10 or 100 μg/kg of the synthetic leu-enkephalin analog dalargin (D-Ala^2-Leu^5-Arg^6-enkephalin) as a single intraperitoneal injection. Control pups were given an equal volume of the solvent (sterile physiological saline). ^{3}H-Thymidine (specific activity 1530 TBq/mol) was injected intraperitoneally in a dose of 1 μCi/g body weight 23 h after dalargin (1 h before euthanasia).

Histotopographic preparations of the heart and tongue were obtained by standard methods [3]. The

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