REVIEWS

DEAR COLLEAGUES !

We are publishing in this issue the review article by I. S. Severina entitled "A soluble form of guanylate cyclase in the molecular mechanism of the physiological effects of nitrogen oxide and in the regulation of platelet aggregation." On the basis of original approaches having no analogs in the Russian and Western literature, the author offers seminal data on the molecular basis for the development of effective new vasodilative and antiaggregating agents. The author's research into molecular mechanisms of directed activation of soluble guanylate cyclase has revealed new enzyme activators taking part in nitrogen oxide generation and regulation of hemostasis and vascular tone in the organism.

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A Soluble Form of Guanylate Cyclase in the Molecular Mechanism of the Physiological Effects of Nitrogen Oxide and in the Regulation of Platelet Aggregation

I. S. Severina

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The article is devoted to the role of heme in soluble guanylate cyclase functioning, the molecular mechanism of nitrogen oxide activation of guanylate cyclase, the role of guanylate cyclase in the process of platelet aggregation, and the mechanism of the antihypertensive and antiaggregative action of certain activators of the enzyme.

Key Words : *guanylate cyclase; heme; nitrogen oxide; human platelets; aggregation*

Guanylate cyclase (GC, E.C. 4.6.1.2) catalyzes the biosynthesis of cyclic 3',5'-guanosinemonophosphate (cGMP), which is a powerful regulator of cell metabolism. There are two forms of the enzyme: soluble and membrane-bound. It is now known that they are not only different proteins, but enzymes with diverse mechanisms of regulation [36]. At the same time, the molecular mechanism of action of these enzymes is unclear. The regulatory mechanism is not fully elucidated; however, there are enough data to conclude that GC is a complex enzyme with a fine and apparently unique

Department of Physiologically Active Peptides and the Guanylate Cyclase System, Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Moscow (10, Pogodinskaya Street, Moscow, Russia, 119832). (Presented by I. P. Ashmarin, Member of the Russian Academy of Medical Sciences)

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regulatory mechanism. The present report is confined to an analysis of the soluble form of GC.

The presence of heme in the molecular structure is a characteristic feature of the soluble form of GC [12]. It is known that the direct precursor of heme in the organism is protoporphyrin IX, which is responsible for GC activation [21]. The introduction of iron into the protoporphyrin ring causes the formation of ferroprotoporphyrin IX , or heme, which inhibits GC activity [20]. This system is thought to play an important role in the endogenous regulation of the enzyme.

The role of heme in soluble GC functioning is usually associated with enzyme activation by endogenous nitrogen oxide (NO) and compounds that produce a NO free-radical group. The therapeutic effects of the most widely used nitrovasodilators (such as nitroglycerin) are due to GC activation and cGMP accumulation. GC activation by NO compounds is associated with interaction between the NO group and GC heme iron, causing the formation of a nitrosyl-heme complex, which is considered to be the true enzyme activator.

The important role of heme in GC regulation has been highlighted by one of the most important discoveries of recent years that has shed new light on the molecular mechanisms of certain cellular physiological processes. This was the determination of the chemical nature of endothelial relaxing factor [27]. It proved to be NO, derived from L-arginine under the action of L-arginine-NO-synthase [26].

Endogenous NO is a powerful factor in hemostasis. It is now considered to be an endogenous vasodilator. Its antihypertensive and antiaggregative properties are linked with direct stimulation of soluble GC by a heme-dependent mechanism and with cGMP accumulation. Thus, GC heme plays a significant role in the vasodilative action of NO compounds and in endogenous regulation of GC functions and vascular tone.

The nature of link between heme and the GC protein molecule is not clearly understood but it is known to be labile, and heme can be split off (for example by lowering the pH, by certain purification methods, etc.), thereby causing some degree of enzyme hemodeficiency [13,37]. Published data concerning the existence of a primary hemodeficient form of soluble GC in living tissues are lacking. At the same time it is obvious that GC hemodeficiency should lead to enzyme endogenous regulation disturbances, to a decreased efficiency of nitrovasodilators, and to vascular tone impairment.

In view of the above, the present review is devoted to a study of the role of heme in soluble GC functioning, the molecular mechanism of NO-dependent GC activation, the role of GC in platelet aggregation, and the antihypertensive and antiaggregative action of certain enzyme activators.

Role of heme in soluble GC functioning. During a comparative analysis of the ability of soluble GC from different sources to be activated by sodium nitroprusside, we could not detect stimulation of GC from rat platelets with this compound. On the other hand, sodium nitroprusside is reported to have caused a 20-30-fold activation of the enzyme from human and rat heart platelets [29]. Possible rat platelet GC hemodeficiency was later confirmed in a more detailed study of this enzyme and also by comparison of its properties with properties of soluble GC from human platelets, whose activity sharply increases in the presence of sodium nitroprusside [29].

According to published data, ion-exchange chromatography is a useful tool for soluble GC purification with simultaneous heme dissociation. Applying this technique we obtained hemodeficient enzyme preparation from human platelets [1]. A characteristic feature of this enzyme preparation is a very low capacity for activation by sodium nitroprusside. However, the addition of other heme-containing proteins (e.g., hemoglobin) to the sample restored this activation. We think that this phenomenon is caused by the additional formation of a nitrosyl-heme complex involving hemoglobin with the subsequent transfer of this complex to the hemodeficient GC, which has a high affinity to the heme-nitrosyl complex [18].

Experiments with GC from rat platelets failed to reveal any sodium nitroprusside activation of GC either before or after ion-exchange chromatography, as well as under various concentrations of thiol and hemoglobin in the sample. These two compounds are known to promote nitroprusside enzyme activation [2].

The data obtained [33] point to the presence of a hemodeficient form of GC in rat platelets and, in contrast to the generally accepted view, lead to the conclusion that heme is not a constituent part of GC. Thus, rat platelets cannot be used as a model system for a study of the influence of NO and NO-forming compounds on platelet GC.

The primary hemodeficiency of GC from rat platelets raised the problem of evaluating of GC heme saturation. A low activatory response of GC from human platelets to NO compounds was also observed in some cases, associated with the diverse individual status of blood donors [14].

An answer to this question can be obtained from our study on the influence of the antioxi-

dant β-alanyl-L-histidine (carnosine) on human platelet GC activity. It is known that soluble GC is activated by unsaturated fatty acids [15], by products of their peroxidation [17] and by free radicals, including hydroxyl radicals [24]. However, the role of antioxidants in GC functioning is still unclear.

Carnosine is a water-soluble antioxidant [9] which is successfully used as a therapeutic agent in the treatment of inflammation, in wound healing, and in the treatment of cataracts [8,25]. The molecular mechanism of action and the biological role of carnosine have not been sufficiently studied.

During a study of the influence of carnosine on human platelet GC we showed that in concentrations that do not alter the basal activity carnosine sharply suppressed (approximately by 70%) sodium nitroprusside-induced GC activation [32]. However, under the same conditions it did not suppress low-level (3-4-fold) activation of hemodeficient GC by sodium nitroprusside [32]. Carnosine also did not suppress protoporphyrin IX-mediated GC stimulation, which is not connected with GC heme [19,32]. The inhibitory effect of carnosine is due to its interaction with GC heme. Thus, carnosine behaves as a specific inhibitor of heme-dependent GC activation by sodium nitroprusside and may be used for estimation of the degree of heme saturation of the enzyme [32]. The experimental results on the effects of carnosine yielded additional data concerning the possible mechanism of soluble GC activation by NO and NO-containing compounds. For example, in experiments with soluble GC from rat heart we obtained data leading to the assumption that enzyme activation by sodium nitroprusside is determined not so much by the presence of heme as by the redox potential of the enzyme molecule. Indeed, whereas in experiments with rat heart GC carnosine suppressed nitroprusside activation only by 33%, in analogous tests with GC from human platelets suppression was 80% [32].

Carnosine also assisted in elucidating the molecular mechanism of the antihypertensive action of sodium nitroprusside. For this purpose the influence of sodium nitroprusside analogs (nitrosocomplexes of transition metals: chromium and cobalt) on soluble GC from human and rat platelets was studied [34]. These complexes differ from sodium nitroprusside in the degree of oxidation of the NO group and in the lack of hypotensive properties. Comparison of the action of these complexes and sodium nitroprusside on the activity of GC from human platelets (heme-containing) and from rat platelets (hemodeficient) showed that the maximal stimulatory effect on human GC (16.2-fold) was caused by sodium nitroprusside. GC from rat platelets was not stimulated by sodium nitroprusside. The studied nitrosocomplexes caused insignificant GC stimulation (4-5-fold) and the degree of activation was practically the same for heme-containing (human platelets) and hemodeficient (rat platelets) soluble GC [34]. These results suggested that the mechanism of GC activation by chromium and cobalt nitrosocomplexes is independent of the enzyme heme, a conclusion which is supported by the fact that hemodeficient GC preparation obtained from human platelets by means of ion-exchange chromatography [1] in 80-90% of cases lost the ability to be activated by sodium nitroprusside. However, the enhancing effect of the above nitrosocomplexes remained practically unchanged [34]. Moreover, carnosine, which specifically inhibits only heme-dependent GC activation [32], reduced the stimulating effect of sodium nitroprusside but had no effect on GC activation by chromium and cobalt nitrosocomplexes [34].

Thus, in contrast to sodium nitroprusside, soluble GC activation by these nitrosocomplexes does not depend on the enzyme heme. On the other hand, the absence of pharmacological effects (antihypertensive action) of the nitroprusside analogs used leads to the conclusion that the heme-dependent mechanism of soluble GC activation is a necessary condition for the realization of the hypotensive action of the transition metal nitrosocomplexes and, possibly, of other NO compounds.

Role of thrombocytic GC in platelet aggregation. It used to be thought that cGMP promotes platelet aggregation [16]. However, it was later found that GC activators, which raise the cGMP level in platelets, also have antiaggregative properties [23]. At the same time, the role of cGMP in this process is not clearly understood, and thus we conducted a study on the model of human platelet aggregation.

The aim of the investigation was to elucidate possible alterations of GC functioning in the aggregation/disaggregation process. For this purpose the model of ADP-induced reversible human platelet aggregation was used. Platelets were obtained from healthy donors. *In vitro* experiments were carried out using a wide range of ADP concentrations (0.5 to 10 μM). Platelet concentration in plasma was 250 mln/ml, allowing only a reversible process to develop [6].

It was shown in an investigation of the dynamics of GC activation by sodium nitroprusside against the background of a 45% level of ADP-induced aggregation that immediately after the in-

duction of aggregation, nitroprusside-induced GC activation began to rise and maximal activation was reached within minutes [6]. Later the reverse process began and the degree of GC activation returned to the initial level. A parallel rise of the platelet cGMP level took place and the attainment of the maximum concentration (3-fold above the baseline) coincided in time with the maximum of the GC nitroprusside activation curve. The cGMP concentration then returned to the basal level. At the same time it was shown that phosphodiesterase activity remained unchanged, so that the rise of the platelet cGMP level was caused by GC activation [6].

It is known that nitroprusside-dependent GC activation is implemented by its NO group, which is responsible for GC heme nitrosylation and causes enzyme activation. Nitroprusside-induced GC activation is enhanced by the addition of hemoglobin (1.5-1.8-fold), this being due (as noted earlier) to additional nitrosyl-heme complex formation and its subsequent transfer to GC with partial hemodeficiency restoration [18]. The stimulating effect of the added hemoglobin began to decrease as the platelet aggregation process developed, totally vanished at the time of maximal GC activation by sodium nitroprusside, and was subsequently restored [30]. Thus, initiation of aggregation was apparently accompanied by increased heme saturation of the enzyme, which could account for the observed augmentation of GC sensitivity to NO and its enhanced response to sodium nitroprusside.

In order to check this assumption, the effects of L-arginine, protoporphyrin IX, and arachidonic acid on GC during the aggregation process were compared with that of sodium nitroprusside. It is known that NO derived from L-arginine activates GC by a heme-dependent mechanism (like sodium nitroprusside). On the other hand, the GC activation mechanism wielded by protoporphyrin IX and arachidonic acid is independent of heme [19,32]. It was shown that immediately after the induction of aggregation the level of GC activation had risen due to the action of not only sodium nitroprusside, but also protoporphyrin IX, arachidonic acid, and L-arginine [30]. Afterward the intensity of stimulation by each activator fell to the background level. These results lead us to speculate that upon the initiation of ADP-induced aggregation the sensitivity of GC to any of the above-listed activators increased independently of the involvement of heme in enzyme activation [30].

The described dynamics of GC activation ability and of the cGMP content in platelets during the aggregation/disaggregation process is much the same on all levels of reversible aggregation. However, a proportionality between the augmentation of reversible aggregation, the increase of enzyme sensitivity, and the cGMP content in platelets was observed only within the range of 40-45% reversible aggregation [28]. Within these limits the aggregation peak coincided with maximum GC activation. Further increase of aggregation did not lead to a parallel increase of stimulator-induced GC activation. Moreover, the GC parameters returned stepwise to the normal level, although the reversible aggregation did not reach its maximum [28]. The same changes of GC parameters were observed in the case of irreversible aggregation. Thus, changes in platelet GC functioning take place at the earliest stages of the aggregation process, i.e., during the first 2-2.5 min [28].

Preincubation of human platelets with sodium nitroprusside (0.1 mM) for 3 min prevented platelet aggregation [28]. Addition of sodium nitroprusside at the 45% peak of reversible aggregation caused platelet disaggregation. The maximal rate of disaggregation coincided with the time of maximal GC activation by sodium nitroprusside [28]. Thus, the efficiency of disaggregation was mainly determined by platelet GC activity and reactivity. Initiation of the aggregation process induced GC activation and promoted cGMP accumulation and inhibition of aggregation. In other words, GC exercises a negative control on platelet aggregation and sends the signal for disaggregation [28].

Augmentation of GC activation ability by any of the used activators regardless of heme presence against the background of platelet aggregation may indicate that the enzyme heme-dependent activatory mechanism is not obligatory for manifestation of antiaggregative (in contrast to antihypertensive) properties by GC activators. Indeed, we have shown [5] that some W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] derivatives activate human thrombocytic GC in low concentrations (0.1-1 μM) and simultaneously cause the depression of ADP-induced reversible aggregation. At the same time, the most effective enzyme activators were found to have the most pronounced antiaggregative effect [5]. The results presented show the relationship between thrombocytic GC function and platelet aggregation ability. Therefore, platelets characterized by heightened aggregation capacity should differ from normal ones in changed GC functioning. Enhanced platelet aggregation ability is described in some diseases. We have studied this problem in human diabetes mellitus.

It was shown that platelets from diabetes patients are more sensitive to ADP by a factor of

1.62 (for type I) and 2.33 (for type II) in comparison with platelets of healthy donors [10]. The platelets of diabetes patients manifested a decreased basal GC activity and reduced ability of the enzyme to be activated by sodium nitroprusside and protoporphyrin IX [7,10]. The reduction of the above-mentioned GC parameters was more marked in type II diabetes mellitus. In other words, differences were found not only between GC functioning in healthy donors and diabetes patients, but, what is of special importance, between various types of diabetes. In light of these data, GC pharmacotherapy may be used for reducing the enhanced capacity of platelet aggregation.

Thus, the adduced data allow us to consider thrombocytic GC as a protective mechanism in the aggregation pathway. Taking into account the fact that the regulatory role of cGMP is manifested only at the early stages of the aggregation process, it should be stressed that new enzyme activators developed on the basis of GC stimulation will be able not only to alleviate platelet hyperaggregation, but also to prevent spontaneous aggregation, thereby avoiding vascular complications. Based on the above data our further investigations were focused on the study of the molecular basis for designing new antihypertensive and antiaggregative agents.

Directed activation of soluble GC as a molecular basis for the development of new vasodilators and antiaggregative agents. The discovery of the endogenous nature of NO sparked a sharp increase in interest in nitrovasodilators. NO formation from nitroglycerin in the biotransformation process [11] attests to the physiological nature of the vasodilative mechanism of action of organic nitrates. In view of this, the search for new GC activators on the basis of chemical structures which can provide for NO production in the body is a promising avenue for creating a molecular basis for the development of new effective vasodilators and antiaggregative preparations.

Thus, we are the first to have studied the new class of Russian-developed compounds, diazetine di-N-oxide derivatives, that can generate NO by a fundamentally new enzyme-independent pathway: NO formation by splitting at physiological pH values and in the absence of thiols [3,22].

In our investigation we found 4 diazetine di-N-oxide derivatives with a strong correlation observed between NO-releasing splitting, activatory action on soluble GC [4], inhibition of human platelet aggregation, and acceleration of disaggregation [31].

Moreover, a study of the spasmolytic activity of these compounds on isolated rat thoracic aorta rings and of their hypotensive effect on spontaneously hypertensive rats anesthetized with urethane showed that there is full correspondence between their splitting ability with NO-release, GC activatory effect, and the above-listed physiological effects [35].

The compound most effective regarding all parameters manifests a spasmolytic action comparable to that of sodium nitroprusside.

The fact that endogenous thiols are not required for NO release from the diazetine 1,2-di-N-oxide derivatives under study makes this class of compounds promising candidates for use in the design of new vasodilative agents. These preparations may be free of such undesirable side effects of modern nitrovasodilators as tolerance development with prolonged administration. Tolerance development is believed to be linked with a deficiency of endogenous thiols.

The antiaggregative properties of the compounds under review show that, taking them as a basis, new preparations can be developed which should be able not only to reduce enhanced platelet aggregation ability but also to prevent spontaneous aggregation and the sequelae of vascular complications.

Thus, the study of the mechanism of directed activation of GC by a new class of compounds which generate NO in biological systems should provide the impetus for the development of a molecular basis for the creation of effective new vasodilative and antiaggregative agents.

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