Vol. 95, No. 2, 1980
July 31, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 529-534

SUPEROXIDE DISMUTASE CATALYZES ACTIVATION OF SYNAPTOSOMAL SOLUBLE GUANYLATE CYCLASE FROM RAT BRAIN

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Received June 6,1980

SUMMARY: Guanylate cyclase in the crude synaptosomal soluble (p_2-soluble) fraction obtained by hypo-osmotic treatment of crude synaptosomal (P_2) fraction from rat brain showed a significantly high activity. The P_2-soluble fraction prepared after incubating (37 °C, 10 min) with membranous fraction of P_2 (P_2-membrane) had a low activity of guanylate cyclase, which was markedly stimulated by superoxide dismutase. The activation by superoxide dismutase was completely inhibited by KCN, β-carotene, Tiron and hemoglobin. The enzyme obtained using gel-filtration chromatography showed no response to superoxide dismutase, but the addition of hemin induced a marked restoration of the responsiveness.

INTRODUCTION

Guanylate cyclase (GC:EC 4.6.1.2) has been reported to be activated by various kinds of free redicals (1). In the previous paper (2), we have reported that the P_2-soluble fraction from rat cerebellum contains a high specific activity of GC, and this enzyme shows a low response to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; one of the most potent activator for GC (3)). In addition it has been also demonstrated that GC activity in the P_2-soluble fraction is markedly inhibited by vitamin A derivatives (4) and hemoglobin (5). Thus, we have suggested that GC in the P_2-soluble fraction of rat cerebellum is activated endogenously through a similar mechanism to the action of MNNG.

In the present study, we have investigated the mechanism of endogenous activation using the P_2-soluble fraction from rat brain on the basis of the responsiveness to free radical scavengers.

MATERIALS AND METHODS

Preparation of P_2-soluble fraction-while brains from male Wistar rats (weighing 200-250 g) were homogenized with 9 vols of 0.32 M sucrose in a glass homogenizer with Teflon pestle. Subcellular fractionation was employed using essentially similar procedures described previously (6). The homogenate was centrifuged

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0006-291X/80/140529-06$01.00/0

Table I

Effect of various compounds reacting with free radicals on GC activity in P_2-soluble fraction from rat brain.

Addition	GC activity (pmoles/min/mg prot ± S.E.)	% of control
None	353 ± 21	100
MNNG*, 1 mM	501 ± 32	142
Retinol, 1 mM	85 ± 7	24
Hemoglobin, 5 µg	42 ± 3	12
β-Carotene, 1 mM	71 ± 5	20
α-Tocopherol, 1 mM	402 ± 50	114
Tiron, 1 mM	74 ± 8	21
SOD**, 5 µg	453 ± 37	128
Mannitol, 10 mM	368 ± 26	104

Each compound was added to the reaction mixture at the final concentration indicated. Each value represents the mean ± S.E. of three separate experiments.
* N-methyl-N'-nitro-N-nitrosoguanidine, ** Superoxide dismutase

fraction was homogenized with ice-cold distilled water (5 ml per g of wet weight), and the treated homogenate (W fraction) was centrifuged at 100,000 X g for 60 min to obtain the precipitate (P_2-membrane) and supernatant (cytosol of P_2; P_2-soluble fraction) fractions, respectively.

Assay of Guanylate cyclase (GC) activity-GC activity was assayed in 20 µl of the reaction mixture containing 50 mM Tris-HCl buffer (pH 7.7), 4 mM $MnCl_2$, 0.1 mM [^{3}H]GTP (diluted to 0.5 Ci/mmol, Radiochemical centre, Amersham), 0.5 mM isobutylmethylxanthine, 10 mM theophylline, 3 mM cyclic GMP, 15 mM creatine phosphate, 20 µg of creatine kinase and the enzyme sample (10-30 µg protein). Enzyme activity was determined by incubatating the reaction mixture for 10 min at 37 °C, and radioactive cyclic GMP formed was separated by thin layer chromatography as previously described (7). Superoxide dismutase (EC 1.15.1.1) from bovine blood, catalase from beef liver and glucose oxidase from Aspergillus Niger were purchased from the Sigma Chemical Co., St Louis, Missouri, U.S.A. Water-insoluble compounds (retinol, β-carotene, α-tocopherol and hemin) were dissolved in dimethylsulfoxide (DMSO) before adding to the assay system. The final concentration of DMSO was less than 1 % which had no significant effect on GC activity. The protein concentration was determined by the method of Lowry et al (8).

RESULTS

As shown in Table I, the GC in P_2-soluble fraction exhibited significantly higher activity than that in original P_2(basal GC activity : 28 ± 0.1 pmoles/min/mg protein), but was poorly stimulated by MNNG. Both hemoglobin and retinol showed a marked inhibition on the GC activity. These results are essentially in agreement with those reported previously (2). Among compounds known as singlet oxygen scavengers tested, β-carotene (provitamin A) significantly supressed the GC activity, whereas α-tocopherol showed no inhibition. Tiron(1, 2-dihydroxybenzene-3,5-disulfonic acid),a superoxide radical scavenger, marked-

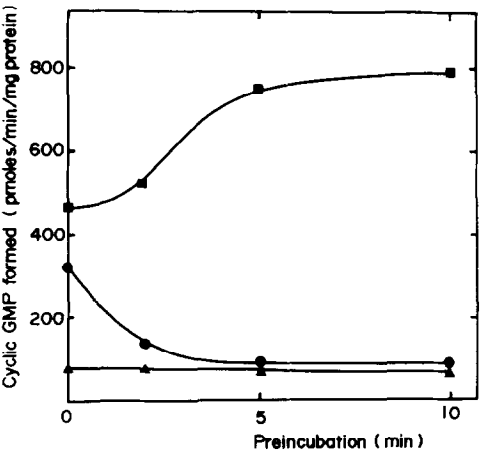

Fig. 1. Alteration of GC activity and responsiveness to MNNG and retinol of GC in P_2-soluble fraction prepared from incubated W fraction:
The W fraction was preincubated at 37 °C for various periods as indicated and subsequently centrifuged to obtain the P_2-soluble fraction. The GC activity in each preparation was determined in the absence (●) and the presence of 1 mM MNNG (■) or 1 mM retinol (▲). Each point represents the mean of three separate experiments.

ly inhibited the GC activity, while superoxide dismutase (SOD) did not suppress but rather stimulated it. The P_2-soluble fraction prepared after preincubating the W fraction for more than 5 min showed significantly lower GC activity than that found in the P_2-soluble fraction prepared without preincubation. Furthermore, the P_2-soluble fraction prepared after incubating the W fraction was strikingly stimulated by MNNG but was not inhibited by retinol ("MNNG-sensitive ") (Fig. 1). When SOD was added to the MNNG sensitive GC preparation, a marked stimulation was observed, and the concentration of SOD required to induce half-maximal activation was 1.1 μg/tube (approx. 165 unit/ml (9)) (Fig. 2: Left). This activation was completely eliminated by using boiled SOD or by adding KCN, an inhibitor for SOD. The P_2-soluble fraction preincubated with SOD for 2 min contained substantial amounts of activated GC even under the condition in which SOD was inhibited by KCN during the incubation for enzymatic assay (Fig. 2 Right). This result suggests that the activator may be effectively maintained in the enzyme preparation. When H_2O_2, a product of catalytic action of SOD, and glucose oxidase (+glucose), as a H_2O_2-generating system, were added to the MNNG-sensitive GC preparation, barely detectable activation of GC was noted

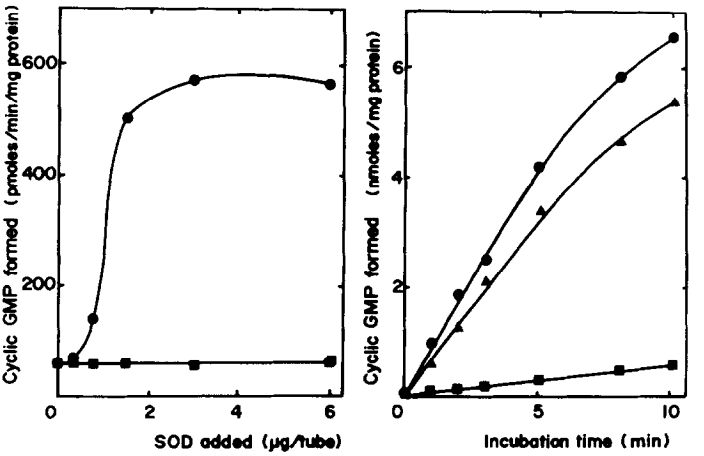

Fig. 2. Activation of MNNG-sensitive GC in P_2-soluble fraction by SOD: The MNNG-sensitive GC was prepared by incubating W fraction for 10 min at 37°C as described in the footnote of Fig. 1. Left: The enzyme activity was assayed in the presence of various concentrations of SOD in the absence (●) and presence of (■) 1 mM KCN. The open square (□) indicates the GC activity measured in the presence of boiled SOD (90 °C, 10 min). Right: GC activity was determined in the presence of SOD (3 µg) without (●) and with (■) 1 mM KCN as a function of incubation time. The closed triangle (▲) indicates the MNNG-sensitive GC activity in P_2-soluble fraction which was preincubated with SOD (3 µg per 12 µg of P_2-soluble protein) at 37 °C for 2 min. The action of SOD was subsequently inhibited by the addition of 1 mM KCN prior to the incubation for the measurement of GC activity. Each point represents the mean of two separate experiments.

Table II

Effect of various compounds reacting with free radicals on the activation of MNNG-sensitive GC by SOD

Addition	GC activity (pmoles/min/mg prot)	
	Mg++	Mn++
None	9	59
H_2O_2, 0.5 mM	22	68
Glucose oxidase, 10 µg + glucose, 1 mM	18	78
SOD, 3 µg	568	786
SOD + mannitol, 10 mM	471	713
SOD + ethanol, 10 mM	328	737
SOD + hemoglobin, 5 µg	12	41
SOD + β-carotene, 1 mM	4	49
SOD + α-tocopherol, 1 mM	408	606
SOD + Tiron, 1 mM	642	33
SOD + Catalase, 10 µg	718	795

MNNG-sensitive GC activity was assayed in the presence of 4 mM $MgCl_2$ or 4 mM $MnCl_2$ as a sole divalent cation. Each compound was added in the reaction mixture at the final concentration indicated. Each value represents the mean of three separate experiments.

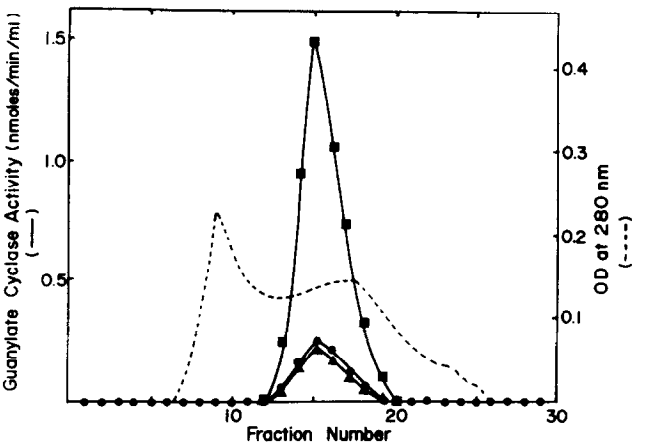

Fig. 3. A typical Gel filtration profile of MNNG-sensitive GC in P_2-soluble fraction:

The P_2-soluble fraction containing MNNG-sensitive GC (5 ml) was precipitated by the addition of 50 % $(NH_4)_2SO_4$ and dissolved with 1 ml of 10 mM Tris-HCl buffer (pH 7.7). Then, the concentrated enzyme (4.2 mg protein) was applied to Sepharose 6B column (1 × 40 cm) equilibrated by 100 mM NaCl/10 mM Tris-HCl buffer (pH 7.7). The column was then eluted with the same buffer, and 1.5 ml fractions were collected. The GC activity in each fraction was assayed in the absence (●) and presence of SOD (3 μg) (▲), or in the presence of SOD and 1 μM hemin + 1 mM ascorbate (■). Neither hemin + ascorbate in the absence of SOD nor ascorbate alone in the presence of SOD showed a significant effect on the GC activity.

(Table II). Either in the presence of Mg^{++} or Mn^{++} as a sole divalent cation, GC was markedly activated by SOD. Mg^{++} was, however, more effective than Mn^{++} in terms of the activation ratio (63- and 13- fold for Mg^{++} and Mn^{++}, respectively). Mannitol and ethanol, hydroxy radical scavengers, showed only a slight inhibition on the SOD-activated GC activity even at a high concentration (10 mM). β-Carotene and hemoglobin completely inhibited the activation by SOD. On the other hand, Tiron suppressed the activation completely in the presence of Mn^{++} but not in the presence of Mg^{++}. Neither catalase nor α-tocopherol showed inhibition on the SOD-activated GC activity. Fig. 3 shows a chromatographic profile of MNNG-sensitive GC in the P_2-soluble fraction using gel-filtration on Sepharose 6B. Although the eluted enzyme showed no response to SOD, the responsiveness to SOD was markedly restored by the addition of a low concentration of hemin.

DISCUSSION

Deguchi (10) reported that the GC in P_2-soluble fraction from brain is endogenously activated by an activator with a low molecular weight. The fact

that the GC in P_2-soluble fraction is inhibited by free radical scavengers suggests the possibility that this endogenous activator may be a kind of free radicals. The activation of GC by SOD was reported previously by Mittal and Murad (11) using a partially purified GC from the rat liver. Although some properties of cerebral GC observed in this study are inconsistent with those previously reported (11), the most distinct difference resides in the requirement for divalent cations; they reported that the activation of GC by SOD was observed in the presence of Mg^{++} but not Mn^{++}, while we observed the activation by SOD in the presence of Mn^{++} as well as Mg^{++}. This discrepancy may be due to the presence of inhibitor(s) or co-factor(s) like hemin in the enzyme preparation they used. In fact, we have found that the enzyme preparations obtained from various organs of rat exhibit a different responsiveness to SOD (unpublished observations). Although detailed mechanisms underlying the activation of GC by SOD unclear at present, it is suggested that free radical(s) generated by a catalytic action of SOD may be capable of activating GC. Moreover, it is noteworthy that the activation of GC by SOD requires a low concentration of a heme derivative which has been reported to have a function as NO radical acceptor for the activation of GC (12). It is likely that there are various kinds of free radicals capable of activating GC, and the GC activity in cerebral P_2-soluble (crude synaptosomal) fraction is physiologically regulated through an endogenous free radical generating system involving SOD.

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