Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 598-606

MODULATION OF RAT MYOMETRIAL GUANYLATE CYCLASE ACTIVITIES BY SODIUM NITROPRUSSIDE AND UNSATURATED FATTY ACIDS

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Received May 11,1979

SUMMARY: Guanylate cyclase activities are present in both soluble and particulate fractions of rat myometrial extract. Triton, slightly stimulated the soluble (50 %) while markedly increasing (1000%) the particulate activity. Both fractions appear to be regulated independently. Predominantly, the soluble form was activated by sodium nitroprusside, involving interactions with SH-groups. On the other hand, the particulate form was stimulated by a series of unsaturated fatty acids and their hydroperoxides. The latter activation appears to result from direct hydrophobic effects rather than peroxide or free radical generation.

INTRODUCTION: In the oestrogen-dominated rat myometrium, a series of physiological contractile effectors including acetylcholine were totally ineffective in altering cGMP levels (1). The rat myometrium appeared to be an exception where stimulation of muscarinic receptors (1,2) or Ca^{+2} influxes (1) does not lead to consistent increases in tissue cGMP content. On the other hand, cGMP levels were markedly enhanced in the presence of hydroxylamine or sodium nitroprusside (SNP) (1).

Current evidence presently indicates that modulation of guanylate cyclase activity may be related to oxidation-reduction or free radical reactions (3-6). Thus, SNP, carcinogenic nitrosamines and sodium azide may activate diverse guanylate cyclases apparently via a common pathway involving the formation of nitric oxide (4). Guanylate cyclase activation by hydroxyl radicals has also been described (6). Although the ability of various fatty acids to stimulate guanylate cyclase from soluble or particulate fractions has, in some cases, been ascribed to direct hydrophobic interactions with the enzyme (7,8), it has also been suggested that activation could result from free radical formation (6) and, in two different systems, activation of

Abbreviations used are: SNP, sodium nitroprusside; DTT, dithiothreitol; BHA, butylated hydroxyanisole.

0006-291X/79/140598-09$01.00/0

guanylate cyclase by unsaturated fatty acids appeared to require their oxidation to peroxides to be effective (9, 10).

The present paper reports on a study designed to evaluate possible mechanisms underlying modulation of soluble and particulate myometrial guanylate cyclase activities.

EXPERIMENTAL PROCEDURE:

- Preparation of tissue and assay of guanylate cyclase activity: Uteri were obtained from immature oestrogen-pretreated rats an myometrial strips were prepared free of endometrium as previously described (11). All experiments were carried out at 0-4°C. Myometrial strips were homogenized with an Ultra Turrax homogenizer in 10 volumes of cold buffer consisting of 0. 25 M sucrose, 5 mM Tris-HCl buffer pH 7. 5, 1 mM EDTA and centrifuged at 700 x g for 5 min. to eliminate cell debris. The pooled 700 x g supernatant fractions constitute the "crude extract" which was further centrifuged at 105. 000 x g for 1 h in order to obtain the particulate and the soluble cell fraction. The pellet was resuspended in a volume of buffer equal to that of the original homogenate.

Guanylate cyclase activity was assayed by a modification of the method of Mittal et al. (4). The standard reaction mixture contained 50 mM Tris HCl buffer pH 7. 5, 0. 5 mM isobutylmethylxanthine, 550 µg creatine phosphate, 33 µg creatine phosphokinase, 1 mM GTP, 1 mM $MnCl_2$ in a final volume of 150 µl. Various agents to be tested were included as indicated. The reaction was initiated by the addition of 10-20 µl of the supernatant or particulate fraction (15-30 µg of protein) and incubation was carried out at 37° for 10 min unless otherwise indicated. Reactions were terminated by the addition of 1. 35 ml of 50 mM sodium acetate buffer pH 4. 0 and heated for 3 min at 90°. cGMP formed was determined in duplicate without purification by the radioimmuno assay (12) as modified by Cailla et al. (13). Protein was determined by the Lowry method (14). Hydroperoxy-derivatives of arachidonic, linoleic and γ-linolenic acids were obtained by catalytic oxygenation of the corresponding fatty acid using Soy bean lipoxygenase (15). The reaction was carried out at room temperature in 0. 5 ml of Tris HCl buffer pH 8. 0 with 600 µM fatty acid and 25 µl of lipoxygenase (5000 u). Measurement of the absorbance at 238 nm indicated that oxygenation was complete between 5 and 10 min.

Material: 3-isobutyl-1-methyl xanthine (Aldrich), Soy bean lipoxygenase (Serva) the various fatty acids (Nuchek Prep Inc.), Creatine phosphate and creatine phosphokinase (Boehringer Mannheim), 2'-O-succinyl cGMP, SNP, DTT, N-ethylmaleimide, BHA and thiourea (Sigma), ^{125}I-succinyl cGMP tyrosine methylester was prepared at URIA, (Dr. F. Dray, Institut Pasteur) and the antibody for radioimmuno-assay of cGMP was from Institut Pasteur Production, France, Indomethacin was kindly supplied by Merck Sharp and Dohme.

RESULTS AND DISCUSSION: The Mn^{+2} dependent guanylate cyclase of the myometrial extract is distributed in both soluble and particulate fractions obtained by centrifugation at 105, 000 x g for 1 h. (Table I). 35 % of the

Table I : Apparent distribution of guanylate cyclase activities in soluble and particulate fractionsof rat myometrium.

	cGMP formed			
	- triton	+ triton	- triton	+ triton
	pmol/min/mg protein		pmol/min/g tissue	
Crude extract	34± 4	231 ± 15	2310 ± 166	15607 ± 1167
105.000 g supernatant	25 ± 3	42 ± 5	870 ± 58	1460 ± 85
105.000 g particulate	46 ± 4	430 ± 20	1513 ± 106	14147 ± 1018

2 g of fresh rat myometrium was homogenized and fractionated as described under "Experimental Procedure". Aliquots (20-30 μg) of each fraction was assayed for guanylate cyclase activity in the absence or presence of 0.1 % triton, with 1 mM $MnCl_2$, 1mM GTP, 10 min at 37° (standard conditions). Results are expressed as total activity, pmol cGMP/min/g tissue; and specific activity, pmol cGMP/min/mg protein. Values are the means ± standard errors of 6 different experiments, each carried in duplicate.

activity was detectable in the soluble and 65 % in the particulate fractions. In the presence of 0.1 % triton, guanylate cyclase activity of the whole extract increased 7-fold. Activity in the pellet was increased 10-fold; by contrast, activity in the soluble fractions was only slightly enhanced (60 %). Thus the high activity observed in crude extracts after treatment with the detergent was attributable to the predominance of particulate guanylate cyclase. Increasing the triton concentration up to 1 % was not accompanied by a further increment in guanylate cyclase activity. As reported for guanylate cyclase activities of different sources (3), both soluble and particulate myometrial preparations preferred Mn^{+2} as sole cation for their activity. With 5 mM Mg-GTP, activity in both fractions was not more than 20 % of that obtained with a maximal effective concentration of 1 mM MnGTP (Table II).

Data of figure 1 illustrate the dose-dependent stimulatory effect of SNP on the soluble activity. In the absence of DTT, maximal stimulation (3-fold) occurred at 50-100 μM SNP; the stimulatory effect then declined with increasing concentrations and reached almost basal values with 1 mM SNP. In the presence of 1 mM DTT, there was a marked enhancement in the SNP effect (6-7 fold), which could be sustained for SNP concentrations as high as 1 mM. It is possible that in the present case, as emphasized for other systems (16) DTT may prevent oxidation of free thiol groups critically

Table II : Effects of MnGTP and MgCTP on basal and stimulated guanylate cyclase activities of soluble and particulate myometrial fractions.

Addition	MnGTP 1mM	MgGTP 5mM
Soluble	pmol cGMP/min/mg protein	
None	25 ± 3	6 ± 2
SNP 100 μM	152 ± 10	130 ± 9
Particulate		
None	44 ± 5	10 ± 2
arachidonic acid 100 μM	200 ± 15	46 ± 5
arachidonic acid 200 μM	80 ± 7	20 ± 3
triton 0.1 %	370 ± 21	45 ± 6
triton + arachidonic acid 200 μM	580 ± 21	578 ± 47

Guanylate cyclase activity was assayed in 105,000 g soluble and particulate fractions either in the presence of 1 mM GTP, 1 mM $MnCl_2$ or 5 mM GTP, 5 mM $MgCl_2$ with or without the addition of SNP, arachidonic acid or/and 0.1 % triton as indicated. 10 min incubation at 37°. Results shown are the means ± standard errors of 3 different experiments each carried in duplicate.

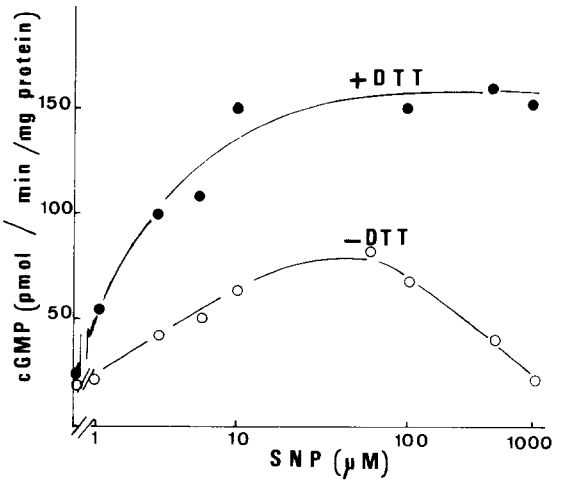

Figure 1 : Dose-dependent effect of SNP on the 105,000 g soluble guanylate cyclase activity of rat myometrium. DTT (1 mM) was absent (o) or present (●) during tissue homogenization, centrifugation and assay which was conducted for 10 min at 37°, in the presence of 1 mM GTP, 1 mM $MnCl_2$ as described under "Experimental Procedure" with the addition of SNP as the indicated concentrations.

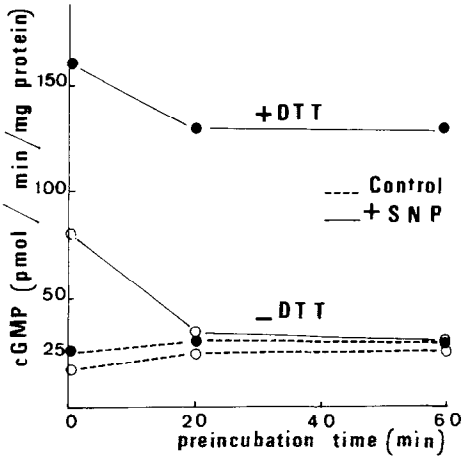

Figure 2 : Effect of preincubation on SNP-induced stimulation of soluble myometrial guanylate cyclase. 20 µg protein of 105,000 g supernatant fraction were preincubated at 37° for the times indicated on the abcissa. DTT 1 mM was present (●) or absent (o) during preincubation and assay as for fig. 1. Interrumpted lines, control activities ; solid lines, addition of SNP (50 µM) for the assay period.

involved in the expression of full enzyme activity. In the absence of DTT and with high concentrations of SNP, additional oxidation of the latter thiol groups may occur, resulting in the complete unmasking of the SNP stimulatory effect. A protective anti-oxidant action exerted by DTT on the soluble myometrial guanylate cyclase is further illustrated in figure 2. Preincubation of the preparation, at 37° in the absence of DTT, resulted in a progressive loss of responsiveness to a subsequent addition of SNP. By contrast, preincubation in the presence of 1 mM DTT resulted in an enzyme activity which was as sensitive to stimulation by SNP as the unpreincubated preparation. The involvement of sulfhydryl groups in the guanylate cyclase activation by SNP was also indicated by the fact that 1 mM N-ethylmaleimide, a SH alkylating agent, completely prevented SNP activation. The stimulatory effect of SNP was less noticeable on the particulate fraction and under optimal conditions was no more than 50 % over basal activity. It was also found that when the total extract was treated with SNP, the stimulated activity predominantly accounted for the soluble preparation. The triton-dispersed particulate preparation was unresponsive to SNP and as for other guanylate cyclases (4) triton inhibited the stimulation of the soluble preparation by SNP.

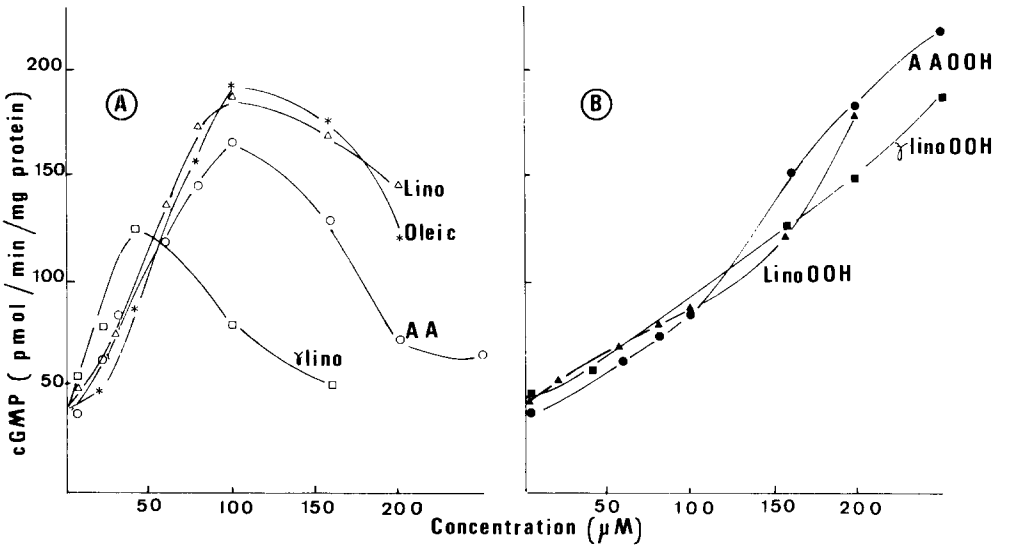

Figure 3 : Dose-dependent sitmulation of particulate guanylate cyclase activity by various fatty acids and hydroperoxides. Guanylate cyclase in the 105,000 g particulate fraction was assayed in the presence of 1 mM GTP, 1mM $MnCl_2$, 10 min at 37° as described under "Experimental Procedure", with the addition of the indicated concentrations (A) of arachidonic (AA), oleic, linoleic (lino), γ-linolenic (γ-lino) acids and (B) of hydroperoxy-arachidonic (AA-OOH), linoleic (lino-OOH) and γ-linolenic(γ-lino-OOH) acids.

The cation requirement for myometrial guanylate cyclase changed with the SNP-activated enzyme, so that Mg^{+2} became as effective as Mn^{+2} as sole cation (Table II). The data of figure 3A show that the particulate activity of the myometrial extract was markedly increased in the presence of a series of unsaturated fatty acids. Activation was dose-dependent, almost, undetectable below 20 μM and reaching a maximum at 100 μM (for γ-linolenic acid, maximal response was attained at 50-80 μM). Increasing the fatty acid concentrations resulted in a progressive loss of activation. The hydroperoxy-derivatives of arachidonic, γ-linolenic and linoleic acids also stimulated particulate guanylate cyclase (figure 3B). The dose-dependent pattern of activation was similar for the various hydroperoxides but appeared strikingly different from that of their corresponding fatty acids. Even though maximal responses attained with the hydroperoxides were similar or even higher in magnitude as compared to the fatty acids and did not decline with increasing concentrations, it is clear that, at low concentrations (20-100 μM) the fatty

acids were consistently more effective than their hydroperoxides. This observation would tend to imply that the fatty acid effects could not be attributed to their conversion to hydroperoxides during guanylate cyclase incubation. The conclusion was strengthened by the fact that DTT (1-5 mM) which inhibits both the lipoxygenase and cyclooxygenase reactions (17) did not appreciably affect the ability of either the fatty acids or the hydroperoxides to stimulate guanylate cyclase. Furthermore, indomethacin (15 μM), a well known inhibitor of the cyclooxygenase (18) failed to prevent guanylate cyclase activation induced by arachidonic acid (not shown). Finally, in the presence of two hydroxyl radical scavengers viz, thiourea and BHA (6, 19) there was no alteration in the fatty acid responses (during 10 min. incubation guanylate cyclase activity was with 300 μM arachidonic acid: 1900 pmol cGMP/mg protein, with arachidonic acid + 5 mM thiourea: 2120 pmol cGMP and with arachidonic acid + 5 mM BHA: 1600 pmol cGMP/mg protein). Similar observations were made with 15 hydroperoxyarachidonic acid, providing evidence that activation of guanylate cyclase in the particulate preparation of rat myometrium by fatty acids and their hydroperoxides did not occur via a mechanism involving the generation of hydroxyl radicals.

Most probably, activation might result from a direct interaction of these lipid components with hydrophobic regions of the particulate guanylate cyclase or associated regulatory units. The difference in the activation patterns between fatty acids and their hydroperoxides may actually reflect differences in the hydrophobic character of the two classes of compounds. Stimulations by arachidonic acid or hydroperoxy arachidonic acid could still be observed in the presence of triton (fig. 4). Greater activity was ultimately achieved with triton + arachidonic acid (or triton + hydroperoxyarachidonic acid) than with either alone. Triton also modified the pattern of fatty acid activation inasmuch as the decline in activity could no more be evidenced with high concentrations of arachidonic acid. It was quite clear that including triton in the assay resulted in complex hydrophobic interactions between the detergent, fatty acids and guanylate cyclase. More important was the observation that guanylate cyclase, when activated in the presence of both triton and arachidonic acid, was able to utilize Mg^{+2} as effectively as Mn^{+2} as sole cation, in contrast to activation induced by either effector alone (Table II). Guanylate cyclase of the soluble fraction was only slightly stimulated by

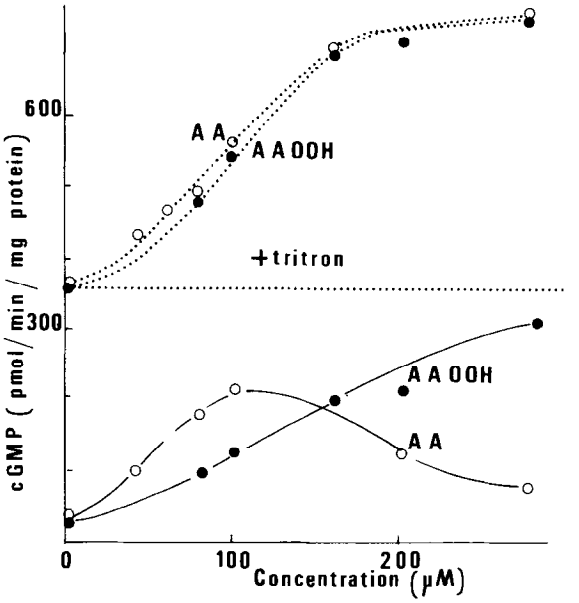

Figure 4 : Effect of arachidonic acid and hydroperoxy arachidonic acid alone and in combination with triton to activate particulate myometrial guanylate cyclase. Assay was conducted as described for fig. 3 in the absence (solid lines) or presence (dotted lines) of 0.1 % triton, with the addition of the indicated concentrations of AA and AA-OOH.

fatty acids or hydroperoxyderivatives . Maximal activation was no more than 50-75 % , similar to the triton effect.

In conclusion, the foregoing results demonstrate the presence in rat myometrial extract of both soluble and particulate guanylate cyclase activities which could be independently modulated. The soluble fraction was the most sensitive to oxidative mechanisms such as those which might be involved in the NO activating pathway (4), and presumably contributed to the stimulatory effect of SNP on myometrial cGMP levels (1). On the other hand, the particulate activity could be markedly enhanced as a result of hydrophobic interactions. Both fractions, when fully activated, utilize a more physiological substrate i. e. MgGTP. It was further demonstrated that the fatty acid activating mechanism operate without the involvement of peroxide derivatives or generation of hydroxyl radicals. It is interesting to note that arachidonic, oleic and linoleic acids (10-3000 μM)were totaly ineffective in raising cGMP levels in intact myometrial strips (data not shown). Under the same conditions, arachidonic acid, via the cyclooxygenase metabolism pathway, has been shown

to induce elevations of tissue cAMP content (11). It is possible to speculate that fatty acids, added in the tissue incubation medium, could not easily reach the guanylate cyclase subcellular sites. Whether tissue guanylate cyclase activity could be modulated by locally liberated fatty acids (e.g. as a result of phospholipase activity) deserves further investigation. It also still remain to be established whether fatty acid activation of guanylate cyclase may share features in common with physiological modulation of rat myometrium cGMP levels, if indeed the latter does occur.

ACKNOWLEDGEMENTS: This work was supported by grants from the CNRS, DGRST, INSERM (C. L. 77. 4. 003. 4) and a contribution from the Fondation pour la Recherche Médicale Française. We are grateful to Mrs J. Robichon and G. Thomas for excellent technical assistance and to Mrs J. Jalicot for typing the manuscript.

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