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SULFHYDRYL GROUP INVOLVEMENT IN THE MODULATION OF GUANOSINE 3′,5′-MONOPHOSPHATE METABOLISM BY NITRIC OXIDE, NOREPINEPHRINE, PYRUVATE AND *t*-BUTYL HYDROPEROXIDE IN MINCED RAT LUNG*

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Abstract—The cyclic GMP content of rat lung mince was increased nearly 50-fold within 4 sec following exposure to nitric oxide. This rapid increase in cyclic GMP accumulation was prevented by 10 mM, but not 1 mM, dithiothreitol which itself caused a slower yet massive (100-fold) increase in the cyclic GMP content of lung mince. Tissue cyclic GMP following nitric oxide exposure declined rapidly even in the presence of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine. The decline in cyclic GMP was accelerated by the thiol oxidant diamide (1 mM). The cyclic GMP content of lung mince was also increased by norepinephrine, pyruvate and *t*-butyl hydroperoxide. Diamide blocked cyclic GMP accumulation in response to these other agents as well as that caused by nitric oxide or dithiothreitol. The results suggest that sulfhydryl group modification may be a common pathway for the enhancement of cyclic GMP synthesis in tissues by a variety of stimuli.

Cyclic GMP formation in tissues is catalyzed by the enzyme guanylate cyclase [GTP pyrophosphate lyase (cyclizing), EC 4.6.1.2]. Tissue cyclic GMP content can change in response to a variety of hormonal [1–3], metabolic [4, 5], and oxidative [6–9] stimuli. An abundance of compelling evidence now suggests that both the soluble and particulate forms of guanylate cyclase are regulated by redox events [10, 11]. Studies from this and other laboratories have indicated that the dynamic modulation of guanylate cyclase activity *in vitro* probably involves the reversible oxidation and reduction of key sulfhydryl groups on the enzyme [8–14]. Of critical importance, however, is the question of whether or not sulfhydryl group modification on the enzyme is the common mediator of changes in tissue cyclic GMP content in response to a variety of stimuli including hormonal, free radical, and metabolic.

The present study was undertaken using minced rat lung to examine the role of sulfhydryl groups in the *in vivo* modulation of guanylate cyclase by various stimuli. Of primary interest were the effects of nitric oxide gas on tissue cyclic GMP accumulation. Nitric oxide is a potent activator of both soluble and particulate guanylate cyclases [12, 15] and has been shown to interact directly with the soluble enzyme purified to homogeneity from liver [15] and lung [16]. A number of *N*-nitroso compounds have been found to stimulate guanylate cyclase and to increase cyclic GMP accumulation in tissues through a mechanism that involves nitric oxide [11]. Nitric oxide has been found to be a useful tool in studies of cyclic GMP metabolism and is perhaps representative of other forms of oxidative activation. In this report, the effects of nitric oxide on cyclic GMP accumulation in lung minces are compared with other stimuli of cyclic GMP in this preparation.

MATERIALS AND METHODS

Male Sprague–Dawley rats (125–150 g) were anesthetized with sodium pentobarbital (30 mg/kg), and the lungs were perfused through the right atrium with 50 ml of ice-cold 0.9% saline. Following complete perfusion, the pure white lungs were removed and placed in Krebs–Ringer bicarbonate buffer at 4°, gassed with 95% O_2–5% CO_2 and containing 1 mg glucose/ml (KRB). Lung minces (0.3 × 0.3 × 0.3 mm), prepared using a McIlwain Tissue chopper, were suspended in KRB at 4°.

For tissue incubation, 100 μl of the freshly prepared tissue suspension containing 1.5 mg tissue protein/ml was transferred to 900 μl of fresh KRB and was preincubated for 20 min with shaking at 37°. Incubations were gassed continuously with 95% O_2–5% CO_2. Following the preincubation, drug and reagent additions were made to the incubation in volumes of 10 μl or less. Nitric oxide gas was delivered directly into the incubation media by the rapid injection of 50 μl of the pure gas, using gas tight syringes. The cyclic GMP and cyclic AMP content of lung minces treated in this manner remained stable for at least 60 min in the absence of any additions.

Cyclic GMP and cyclic AMP were determined by radioimmunoassay as described [12, 15]. The incubation was terminated by the rapid addition of 0.5 ml of ice-cold 20% trichloroacetic acid to the sample

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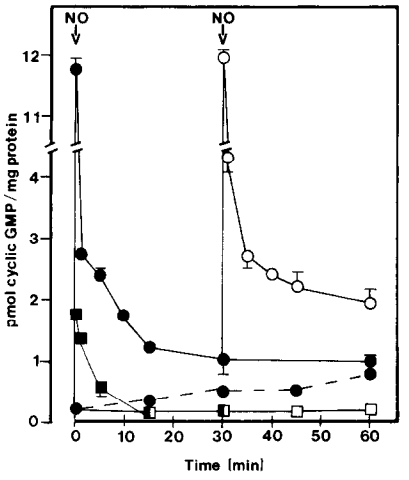

Fig. 1. Effects of nitric oxide on the cyclic GMP levels of rat lung mince. Lung minces, prepared and preincubated as described in Materials and Methods, were incubated for 60 min in the absence (□——□) or presence (●- - -●) of 0.2 mM MIX. At the times indicated, some incubations were terminated and assayed from cyclic GMP. At time zero, 50 μl of nitric oxide gas was introduced into some incubations in either the absence (■——■) or presence (●——●; ○——○) of 0.2 mM MIX. Some incubations were exposed to a second 50 μl dose of nitric oxide at 30 min (○——○). The earliest time after nitric oxide exposure at which an incubation was terminated was 4 sec.

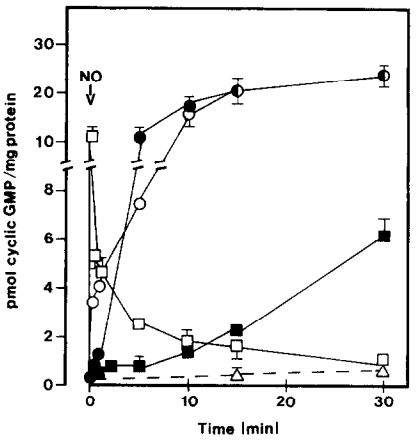

Fig. 2. Effects of dithiothreitol on cyclic GMP accumulation in rat lung mince. Lung minces, prepared and preincubated as described in Materials and Methods, were incubated for 30 min in the presence of 0.2 mM MIX in the absence (△- - -△) or presence of other additions. At the times indicated, some incubations were terminated and assayed for cyclic GMP. At time zero, 1 mM dithiothreitol (■——■), 1 mM dithiothreitol plus 50 μl nitric oxide (□——□), 10 mM dithiothreitol (●——●), or 10 mM dithiothreitol plus 50 μl nitric oxide (○——○) was added to some incubations. The earliest time after nitric oxide at which an incubation was terminated was 4 sec.

which was immediately homogenized for 5 sec using a Polytron homogenizer. The homogenate was centrifuged at 4000 *g*, and the resulting supernatant fraction was extracted three times with 5 vol. of H_2O saturated ether. The extract was then acetylated, if necessary, and the cyclic nucleotide content was determined. Proteins were determined on the trichloroacetic acid pellet following dissolution in 1 N NaOH by the method of Lowry *et al.* [17]. All results are expressed as pmoles cyclic GMP or AMP/mg protein and are the means ± S.E. of triplicate incubations from representative experiments. All results presented were confirmed in at least three separate lung preparations.

All materials were obtained as described previously [9, 12, 15].

RESULTS

The cyclic GMP content of rat lung mince did not change during a 60-min incubation at 37°. The introduction of a maximal concentration of nitric oxide gas into the incubation (Fig. 1) caused an immediate increase in the cyclic GMP content of rat lung mince within 4 sec after exposure that was enhanced by 0.2 mM 1-methyl-3-isobutylxanthine (MIX). Following this sharp nitric oxide-induced rise, the cyclic GMP content fell rapidly at first and then more slowly over 30 min. Although cyclic GMP levels declined after nitric oxide, even in the presence of MIX, they did not return to control levels if the phosphodiesterase inhibitor was present. The loss of lung cyclic GMP with time was probably not related to the irreversible inactivation of tissue guanylate cyclase since re-exposure to a second maximal dose of nitric oxide resulted in a secondary response. It is interesting to note that the reduction in cyclic GMP content with time after the second nitric oxide exposure was somewhat slower than that following the initial exposure despite the fact that the initial responses were identical. The second exposure to nitric oxide also caused cyclic GMP levels to return to a new, higher basal level.

Dithiothreitol caused a dose- and time-dependent elevation in the cyclic GMP content of lung minces (Fig. 2). Although the addition of 1 mM dithiothreitol did not alter the response of the tissue to nitric oxide, 10 mM dithiothreitol prevented the rapid response of cyclic GMP to nitric oxide. The stimulation of mince cyclic GMP levels by dithiothreitol was enhanced by 0.2 mM MIX (Table 1). The data suggest that dithiothreitol caused a time-dependent activation of tissue guanylate cyclase.

Table 1. Effects of MIX on cyclic GMP accumulation in response to dithiothreitol*

Addition	Cyclic GMP (pmoles/mg protein)	
	MIX (0.2 mM)	
	−	+
Dithiothreitol (1 mM)	1.29 ± 0.06	3.22 ± 0.71
Dithiothreitol (10 mM)	7.60 ± 1.08	24.83 ± 2.12

* Rat lung mince was incubated for 15 min in either the absence or presence of 0.2 mM MIX and the concentration of dithiothreitol indicated. Values are means ± S.E. of triplicate incubations from representative experiments.

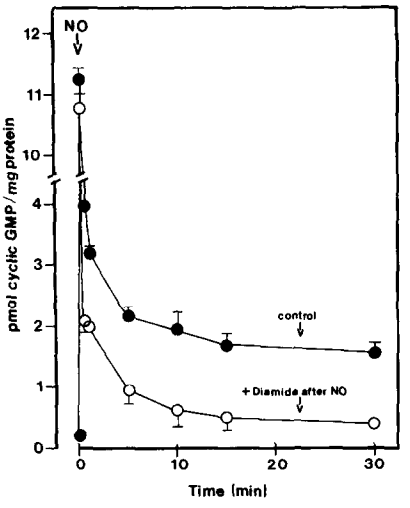

Fig. 3. Effects of diamide on the response of lung mince cyclic GMP to nitric oxide. Lung minces, prepared and preincubated as described in Materials and Methods, were incubated for 30 min in the presence of 0.2 mM MIX after exposure to nitric oxide at time zero (●——●). Diamide (final concentration 1 mM) was added to some incubations immediately after nitric oxide (○——○). At the times indicated, incubations were terminated with the earliest at 4 sec following nitric oxide.

Recent studies from this laboratory have shown that the thiol oxidant diamide can accelerate the decay of nitric oxide-stimulated soluble or particulate guanylate cyclase ([12] and J.M. Braughler, manuscript in preparation). Diamide (1 mM) added immediately after nitric oxide exposure had a similar effect on the cyclic GMP levels of lung mince following nitric oxide (Fig. 3).

In addition to enhancing the decline of nitric oxide-stimulated tissue cyclic GMP levels, diamide also blunted the response of cyclic GMP to nitric oxide (Table 2). Although 1 mM diamide itself had little effect on basal cyclic GMP levels, it also significantly reduced the response of lung mince cyclic GMP to maximal concentrations of norepinephrine, pyruvate and *t*-butyl hydroperoxide. Catecholamines and pyruvate have been shown to alter the cyclic GMP content of several tissue preparations in a dose-dependent manner [1, 4, 18, 19]. The organic hydroperoxide, *t*-butyl hydroperoxide, at 10 μM was found to increase significantly cyclic GMP levels during a 1-min incubation. This effect was not surprising since fatty acid hydroperoxides and prostaglandin endoperoxides can activate guanylate cyclase [8]. Although the cyclic GMP accumultion caused by 1 mM dithiothreitol took some time to develop, it too was blunted by 1 mM diamide. Diamide had no effect on the response to 10 mM dithiothreitol.

With the exception of norepinephrine, none of the agents tested had any effect on cyclic AMP accumulation in lung minces. Diamide (1 mM) did not affect basal cyclic AMP levels; however, it did slightly reduce the cyclic AMP response to 100 μM norepinephrine, though not to the extent seen for cyclic GMP (Table 3). Diamide apparently did not inhibit basal guanylate or adenylate cyclase activity *in vivo* since it did not reduce basal cyclic GMP or cyclic AMP levels during a 30-min incubation in the absence of MIX (data not shown).

Other blocking drugs, including indomethacin (10 μM), nifedipine (10 μM) and propranolol (1 μM), were also tested for their abilities to inhibit cyclic GMP accumulation by the various stimuli examined in Table 1. Of these, only propranolol had a

Table 2. Effects of diamide on cyclic GMP accumulation in lung mince in response to various stimuli*

Addition	Cyclic GMP (pmoles/mg protein)		
	Diamide (1 mM)		
	−	+	+/−
Control	0.35 ± 0.08	0.31 ± 0.11	0.89
Nitric oxide (50 μl)	3.63 ± 0.75†	1.13 ± 0.30†‡	0.31
Norepinephrine (100 μM)	2.40 ± 0.48†	0.94 ± 0.24†‡	0.39
Pyruvate (1 mM)	2.10 ± 0.38†	0.73 ± 0.03†‡	0.34
t-Butyl hydroperoxide (10 μM)	2.13 ± 0.37†	0.41 ± 0.04‡	0.19
Dithiothreitol (1 mM)	2.27 ± 0.22†	1.16 ± 0.21†‡	0.50
Dithiothreitol (10 mM)	23.70 ± 0.81†	24.12 ± 1.36†	1.01

* Rat lung mince was incubated for 5 min after the addition of 0.2 mM MIX in either the absence or presence of 1 mM diamide. The additions indicated were then made, and the incubation was terminated after 1 min (control, nitric oxide, norepinephrine, pyruvate, *t*-butyl hydroperoxide) or 15 min (dithiothreitol). Values are means ± S.E. of triplicate incubations from representative experiments.

† Significantly different from control without diamide ($P < 0.01$).

‡ Significantly different from addition without diamide ($P < 0.01$).

Table 3. Effects of diamide on cyclic AMP accumulation with norepinephrine*

Addition	Cyclic AMP (pmoles/mg protein)		
	Diamide (1 M)		
	−	+	+/−
Control	7.4 ± 0.7	7.8 ± 1.0	1.05
Norepinephrine (100 μM)	41.1 ± 5.2†	34.7 ± 2.6†‡	0.84

* Conditions were identical to those described in Table 1. Values are means ± S.E. of triplicate incubations from representative experiments.

† Significantly different from control without diamide ($P < 0.01$).

‡ Significantly different from addition without diamide ($P < 0.05$).

significant inhibitory effect, reducing the cyclic GMP response to norepinephrine by 20%.

DISCUSSION

Recent studies from this and other laboratories have demonstrated that enzyme sulfhydryl groups play a critical role in the regulation of guanylate cyclase activity. Brandwein *et al.* [20] and Tsai *et al.* [21], for example, have found that the formation of mixed disulfides or sulfhydryl group oxidation results in the inactivation of guanylate cyclase. Mixed disulfide formation also reduces the responsiveness of guanylate cyclase to activation by agents such as nitric oxide [20]. Studies in this laboratory with the particulate [12] and purified soluble enzymes from rat lung (J. M. Braughler, manuscript in preparation) have indicated that the dynamic regulation of guanylate cyclase by nitric oxide involves the reversible oxidation and reduction of key sulfhydryl groups on the enzyme.

In the present study, the cyclic GMP content of rat lung mince was found to increase rapidly upon exposure to nitric oxide. This almost instantaneous response to nitric oxide is similar to that observed in the intact breathing lung to inhaled nitric oxide gas or tobacco smoke ([22] and M. B. Maron, T. D. Crowe and J. M. Braughler, manuscript submitted for publication). These findings support the contention that guanylate cyclase is exquisitely sensitive to oxidative events.

The nitric oxide-elevated cyclic GMP content of lung mince declines with time even in the presence of phosphodiesterase inhibition. In all probability, the large and rapid increase in cyclic GMP caused by nitric oxide was able to overcome the inhibition of phosphodiesterase by MIX. This would result in the enzymatic hydrolysis of cyclic GMP until its concentration was no longer sufficient to surmount the competitive phosphodiesterase inhibition by MIX and a new basal level would be attained (Fig. 1). Also since nitric oxide is a gas and was probably rapidly lost from the incubation, the source of guanylate cyclase activation would disappear with time. Thus, the rate of cyclic GMP synthesis would slow as nitric oxide-activated enzyme returned to its basal state and would no longer be sufficient to cause continued accumulation of cyclic GMP. When the source of guanylate cyclase stimulation was continuously present as seen with dithiothreitol, on the other hand, cyclic GMP synthesis exceeded hydrolysis and net accumulation occurred.

Dithiothreitol accelerates the reversal of nitric oxide-stimulated guanylate cyclase *in vitro* probably by mediating the reduction of enzyme sulfhydryl groups oxidized by nitric oxide. In addition, dithiothreitol can prevent the loss of enzyme activity by excessive oxidation [12]. The fact that 10 mM dithiothreitol reduced the peak response of lung mince to nitric oxide suggests that it may have prevented or blunted the oxidation of enzyme sulfhydryl groups by nitric oxide *in vivo*. This is consistent with studies that have shown that high concentrations of thiols may block activation of guanylate cyclase by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [13] and fatty acid hydroperoxides [8]. The large accumulation of cyclic GMP caused by 1 mM, and particularly 10 mM, dithiothreitol may, in part, be due to the *in vivo* formation of free radical thiol intermediates [23] which would activate the guanylate cyclase. Stimulatory effects of thiols on guanylate cyclase *in vitro* have been reported [24]. The observation that dithiothreitol did not enhance the *in vivo* cyclic GMP response to nitric oxide, as it does the particulate or purified soluble guanylate cyclase [12, 14, 25], is consistent with *in vitro* studies by Ignarro *et al.* [26] utilizing the crude soluble enzyme.

The accelerated reduction of mince cyclic GMP content by diamide following nitric oxide exposure is similar to its effects on nitric oxide-stimulated guanylate cyclase ([12] and J. M. Braughler, manuscript in preparation). In those studies diamide was found to markedly accelerate the inactivation of nitric oxide-stimulated enzyme. In the present study, diamide added after nitric oxide may have accelerated the already rapid inactivation of tissue guanylate cyclase following nitric oxide exposure.

Diamide, oxidized glutathione, and other thiol oxidants have been shown to inhibit guanylate cyclase activity and block enzyme activation by *N*-nitroso and other oxidative compounds [12]. Diamide was found to blunt *in vivo* cyclic GMP accumulation in lung minces in response to nitric oxide, norepinephrine, pyruvate, *t*-butyl hydroperoxide, and dithiothreitol. Such an observation suggests that all of these stimuli ultimately mediate the *in vivo* activation of guanylate cyclase via a common mechanism. Whereas nitric oxide is believed to interact directly with guanylate cyclase to increase cyclic GMP in tissues [15], cyclic GMP accumulation caused by hormones [1–3, 18, 19] or metabolic events [4] probably is, by an indirect mechanism requiring Ca^{2+}. Recent studies by Spies *et al.* [27] and Briggs and DeRubertis [28] have suggest that hormonally induced cyclic GMP accumulation in tissues is linked to the Ca^{2+}-dependent release of fatty acids and their subsequent oxidation to fatty acid peroxides. This is an attractive hypothesis since, as demonstrated in the present study and by others [8], fatty acid hydroperoxides can activate guanylate cyclase and increase the cyclic GMP content of intact tissues (Table 1).

Since diamide did not reduce the basal cyclic GMP content of lung mince incubated in the absence of MIX, it would appear that, under the conditions of this study, sulfhydryl group modification by diamide interfered only with the *in vivo* activation of guanylate cyclase. The fact that cyclic AMP accumulation in response to norepinephrine was not as sensitive to diamide suggests that the effect of diamide was relatively specific for guanylate cyclase. It is clear from these observations that sulfhydryl group modification *in vivo* is in some common way responsible for the enhanced synthesis of cyclic GMP in tissues in response to various stimuli. Additional *in vivo* studies are now needed to more precisely define the various processes involved in the regulation of cyclic GMP metabolism by drugs, hormones, metabolic events, and redox.

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