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ARREST OF MAMMARY TUMOR GROWTH IN VIVO BY L-ARGININE: STIMULATION OF NAD-DEPENDENT ACTIVATION OF ADENYLATE CYCLASE

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SUMMARY: In vivo growth of hormone-dependent rat mammary tumors was arrested by daily injections of L-arginine (L-arginine·HCl 50 mg/200g rat s.c.). Arginine + $N^6,O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate (DBcAMP) acted synergistically to enhance the growth inhibitory effect. Growth arrest by arginine was accompanied by a sharp increase in cellular cAMP content, which was preceded by parallel increases in NAD-dependent ADP-ribosylation of the membrane proteins and NAD-dependent activation of adenylate cyclase. The ADP-ribosylation of the membrane proteins required GTP and was catalyzed similarly by the 105,000 x g supernatant fraction of the tumor and by cholera toxin. These results suggest a specific role for arginine in the cAMP-mediated inhibition of mammary tumors.

INTRODUCTION: Previous studies from this laboratory showed that $N^6,O^{2'}$-dibutyryl cyclic adenosine 3',5'-monophosphate (DBcAMP) inhibits growth of mammary carcinomas in the rat (1). We now show that L-arginine inhibits in vivo growth of two hormone-dependent rat mammary tumors, primary, 7,12-dimethylbenz (α) anthracene (DMBA)-induced (2) and transplantable MTW9 (3). Evidence is presented to show that growth inhibition by arginine is accompanied by a sharp increase of cellular cAMP content and NAD-dependent activation of adenylate cyclase and that arginine exhibits a synergistic effect with DBcAMP in vivo.

RESULTS AND DISCUSSION: Daily injections of L-arginine into rats bearing DMBA tumors consistently produced growth inhibition of tumors. At a dose of 50 mg arginine·HCl/200 g rat s.c./day, growth inhibition was observed within a few days and within 2 weeks, tumor size was reduced to 80% of the initial size (Fig. 1A). The growth inhibition was dose-dependent. Tumor-bearing animals showed no toxic effects, as evaluated by body weight and food consumption, even

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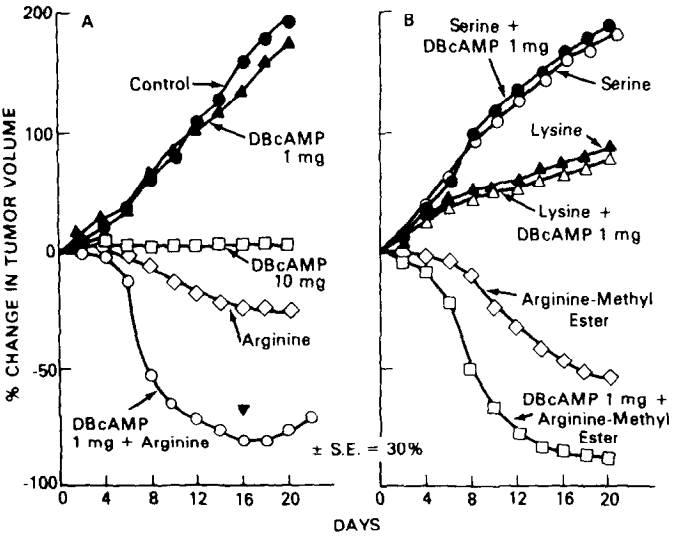

Fig. 1. Syngergistic action of arginine and DBcAMP on DMBA tumor growth inhibition in vivo. Control, no treatment. Dibutyryl cAMP (10 mg or 1 mg), L-arginine·HCl (50 mg), L-lysine·HCl (50 mg), L-serine (50 mg) and L-arginine methyl ester·2HCl (10 mg) were given in 0.2 ml of 0.85% NaCl solution/day/200g rat s.c. Values represent an average of 20 tumors for each group (± S.E. = 30%). All injections were given at a site distant from the tumor. Tumor volume was calculated as previously described (1). ▼, stop injection.

when injected with 100 mg/200 g rat s.c./day for 20 days (data not shown). Arginine + DBcAMP, when given as a combined dose, acted synergistically to produce a striking inhibition of tumor growth. Dibutyryl cAMP alone, at a dose of 10 mg/200 g rat s.c./day, produced growth arrest but one-tenth of this dose had no appreciable effect [Fig. 1A (1)]. When DBcAMP, at a dose of 1 mg/200 g rat s.c./day, was injected together with arginine, however, growth inhibition was greatly enhanced: tumors regressed to 50% of their initial size within 1 week (Fig. 1A) and after 2 weeks the majority of tumors decreased to 10% of their initial size. Upon cessation of this treatment, tumors resumed growth, indicating that the synergistic effect of DBcAMP and arginine is reversible (Fig. 1A). Regression of the hormone-dependent, transplantable mammary tumor, MTW9, was also produced with this treatment but no inhibitory effect by arginine + DBcAMP was observed with DMBA #1 (4) and MT13762 (5) mammary tumors (data not shown). The latter result was consistent with the lack of response of these hormone-independent tumors to hormone-removal (ovariectomy) or DBcAMP treatment (4).

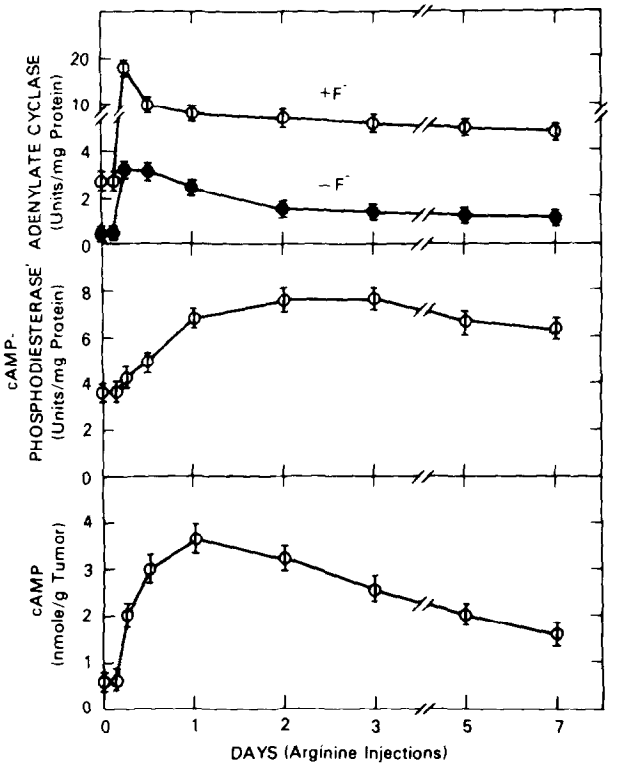

Fig. 2 Increases of adenylate cyclase, cAMP-phosphodiesterase activities and cAMP content of DMBA tumors in response to arginine treatment. Tumors were removed at times indicated and homogenized with 2 volumes of 50 mM Tris-HCl buffer, pH 7.5. Adenylate cyclase was assayed by measuring the conversion of 5'-adenyl-yl[^{3}H]imidodiphosphate (8) to [^{3}H]cAMP in homogenates by the method of Krishna et al. (9) in ± NaF as previously described (11). Cyclic AMP-phosphodiesterase activity was measured in homogenates using the 2-step assay system of Thompson and Appleman (10) at low cAMP concentration, 0.125 M as previously described (11). Cyclic AMP levels in tumors were measured by radioimmunoassay using the acetylation procedure (12, 13) as described by Collaborative Research Inc., Waltham, Mass. Data represent the mean of 10 tumors ± S.E. (bars). Protein concentrations were measured by the method of Lowry et al. (14).

The synergistic growth inhibitory effect of DBcAMP and arginine was also observed when the arginine analogue, arginine methyl ester, was substituted at a dose of 10 mg/200 g rat s.c./day (Fig. 1B). However, when other amino acids, such as lysine or serine, were combined with DBcAMP, growth inhibition was not enhanced, although a 50% decrease in the tumor growth rate was observed with lysine alone (Fig. 1B). These results suggest that arginine may be specifically involved in the cAMP-mediated growth inhibition of mammary tumors.

We therefore measured the level of cAMP and the activities of adenylate cyclase (6) and cAMP-phosphodiesterase (7) in tumors during arginine treatment

of the host. As shown in Fig. 2, within 1 day following the initial arginine injection, the cAMP content of tumors increased 6-fold and remained high (2-fold over the control value). The increase in cAMP content followed a sharp increase in adenylate cyclase activity of the tumors: the basal and F^--stimulated activities (13) increased 7-fold and 6-fold, respectively, within 6 h post-injection (Fig. 2). A 2-fold increase in cAMP-phosphodiesterase activity also occurred within 1 day post-injection (Fig. 2). The level of cAMP and the activities of adenylate cyclase and phosphodiesterase subsided in tumors that resumed growth after cessation of arginine treatment (data not shown).

Results of previous studies *in vitro* suggested that arginine may be involved in the NAD-dependent activation of adenylate cyclase. Cholera toxin was shown to catalyze the hydrolysis of NAD and the transfer of ADP-ribose from NAD to the guanidino group of arginine (15). Thus ADP-ribosylation of an arginine or similar amino acid residue in the acceptor protein of the adenylate cyclase system may be catalyzed by cholera toxin *in vivo* (16). Evidence that mammalian cells might utilize a similar mechanism for the physiologic activation of adenylate cyclase was demonstrated by the isolation of an avian erythrocyte soluble protein having ADP-ribosyltransferase activity just as cholera toxin (17).

We examined whether the increase of adenylate cyclase activity found in DMBA-induced tumors during arginine treatment correlated with the NAD-dependent activation of adenylate cyclase. The soluble fraction (105,000 x g supernatant, S_3) from DMBA tumors in the presence of GTP (18) enhanced the incorporation of [adenine-^{14}C]NAD into the tumor membrane as trichloroacetic acid-precipitated protein (ADP-ribosyl protein, Table I). Cholera toxin in the presence of GTP also catalyzed the NAD-dependent ADP-ribosylation of the membrane proteins (Table I) but did not show an additive effect when combined with the S_3 (data not shown). The ADP-ribosylation of the membrane proteins catalyzed by either S_3 or cholera toxin was markedly inhibited in the presence of arginine methyl ester (Table I). These data agree with the findings that arginine methyl ester inhibits the transfer of ADP-ribose to the acceptor proteins catalyzed by either cholera toxin or avian erythrocyte protein

Table I

Parallel Increases in NAD-dependent ADP-ribosylation and NAD-dependent Adenylate Cyclase Activation in DMBA Tumor Membranes Following Arginine Methyl Ester Treatment In Vivo

Treatment In Vivo	Additions to Assay	ADP-ribosyl Protein (cpm/mg membrane protein)	Adenylate Cyclase Activity (pmol of cAMP/mg membrane protein/h)
None	None	195	1.5
	S_3	200	1.4
	S_3 + GTP	520	6.5
	CT* + GTP	500	6.4
	S_3 + GTP + Arginine methyl ester	210	1.8
Arginine methyl ester	None	202	2.7
	S_3	208	2.5
	S_3 + GTP	1500	23.0
	CT* + GTP	1420	20.5
	S_3 + GTP + Arginine methyl ester	217	2.5

Tumors removed from control and treated hosts (3 h after L-arginine methyl ester injection, 10 mg/200g rat s.c.) were homogenized with 5 volumes of 50 mM potassium phosphate + 0.25 M sucrose, pH 7.0. The homogenates were centrifuged at 770 x g for 10 min and the resulting supernatants were centrifuged at 27,000 x g for 10 min. These supernatants were centrifuged at 105,000 x g for 60 min and the resulting supernatant fractions were used as S_3. The pellets centrifuged at 27,000 x g were resuspended in 20 volumes of 50 mM potassium phosphate + 1 mM EDTA, pH 7.0 and centrifuged at 27,000 x g for 10 min. The suspension in the potassium phosphate buffer in the absence of EDTA and centrifugation of the pellets were repeated twice. The final pellets suspended in 50 mM potassium phosphate, pH 7.0 (1.0 ml/g original tumor) were used as the membrane preparations (~10 mg protein/ml). ADP-ribosyltransferase assays followed the method of Moss and Vaughan (17). The assay mixtures prepared in a total volume of 0.4 ml containing 50 mM potassium phosphate buffer (pH 7.0) consisted of: 30 μM [adenine-^{14}C]NAD (265 mCi/mmole); membranes (0.5 - 2.0 mg protein); GTP, 10^{-4}M; CT*, activated (17) cholera toxin 13 μg or S_3(0.25 - 1.0 mg protein); ± arginine methyl ester, 75 mM. After incubation at 30°C for 45 min, the reaction was stopped by the addition of 3 ml cold 50 mM potassium phosphate buffer and centrifuged at 27,000 x g for 10 min. The membranes were washed 3 times with potassium phosphate buffer, resuspended in 1 ml of the buffer to which 1 ml cold 20% TCA was then added. After 30 min at 0°C, the samples were filtered through 0.45 μm Millipore filters, washed 5 times with 2 ml cold 5% TCA and counted by liquid scintillation. The same conditions were used to measure the NAD-dependent activation of adenylate cyclase in the membranes except that [adenine-^{14}C]NAD was replaced by 5 mM NAD. The final washed membranes were assayed for adenylate cyclase activity in the absence of NaF (see the legend to Fig. 2). Each value is the average of triplicate determinations; the standard error was less than 7 percent. The data represent one of several experiments that gave essentially the same results.

(17). Thus the soluble supernatant fraction of DMBA tumors seems to possess ADP-ribosyl transferase activity just as cholera toxin and avian erythrocytes. Both S_3 and cholera toxin activated adenylate cyclase in the membrane (Table I) and the activation was dependent on NAD, just as that produced by cholera toxin or avian erythrocyte protein in other systems (17, 19, 20).

Within 3 h after arginine methyl ester treatment in vivo, parallel increases (~3-fold) in NAD-dependent ADP-ribosylation of the tumor membrane proteins and adenylate cyclase activation were observed (Table I). These activities were strongly inhibitd when arginine methyl ester was added to the incubation medium (Table I). It has been shown that arginine methyl ester inhibits the ADP-ribosylation of acceptor proteins by acting as an alternative acceptor, forming ADP-ribose-arginine methyl ester (15). Thus the site of interaction of ADP-ribose-acceptor protein with the transferase may contain an arginine moiety.

It seemed likely that the increase of the ADP-ribosylation and adenylate cyclase activation following arginine methyl ester treatment in vivo might result from an increase in ADP-ribose-acceptor protein in the tumor membrane. This possibility was tested indirectly by incubating the S_3 from control and treated tumors with either homologous or heterologous membrane preparations. Indeed, we found that both S_3 from control and treated tumors exerted a similar degree of ADP-ribosylation and adenylate cyclase activation on the membrane preparations (data not shown). It appears, therefore, that arginine methyl ester treatment in vivo enhances membrane acceptability of ADP-ribosylation but it does not affect the transferase activity of the soluble fraction.

Several earlier studies have shown that arginine inhibits in vivo growth of various experimental tumors (21-25) as well as the tumorigenicity of carcinogen-induced tumors (26).

We now show that growth inhibition of mammary tumors by arginine is accompanied by sharp increases in adenylate cyclase activity and cellular cAMP content. We have also observed similar activation of the cAMP system concomitant with inhibition of cell replication in arginine-treated human breast cancer cells (MCF-7) in culture (Cho-Chung et al., in preparation). It is, therefore, likely that, in vivo, arginine exerts its effect directly on the tumor cell.

Demonstration of the enhancement of tumor growth inhibition by the synergistic action of arginine and DBcAMP suggests that these agents have distinct roles in this process: DBcAMP may act intracellularly to induce cAMP-dependent protein

kinase (27, 28), whereas arginine appears to act by increasing the cellular cAMP content via activation of adenylate cyclase in the tumor membrane.

The presence in the tumors of a soluble protein that activates adenylate cyclase and possesses ADP-ribosyl transferase activity supports the earlier proposal (17) that mammalian cells might utilize a similar mechanism as cholera toxin for physiologic activation of adenylate cyclase. The increased or decreased cellular cAMP levels found in neoplastic tissues (29) might, therefore, result from an abnormality in either the soluble protein (ADP-ribosyl transferase) or ADP-ribose-acceptor proteins in the membrane.

The mechanism by which arginine treatment *in vivo* causes the stimulation of NAD-dependent ADP-ribosylation and adenylate cyclase activation in the membrane is not yet understood. Studies on the characterization of the ADP-ribosyl protein of the tumor membrane are underway and should help to elucidate the key role that arginine may play in cAMP-mediated growth inhibition *in vivo*.

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