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Increased incorporation of adenosine into adenine nucleotide pools in serum-deprived mammalian cells

(growth regulation)

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ABSTRACT **The effects of serum deprivation on the incorporation of adenosine into the intracellular adenine nucleotide pools by several mamalian cell lines were studied. Cells arrested in the G_1 phase of the cell cycle showed increased incorporation of exogenous adenosine into their adenine nucleotide pools as compared with growing cells. This phenomenon is unexpected because salvage pathways from all other preformed nucleosides and bases as well as the *de novo* synthesis of adenine nucleotides is decreased after arrest of growth by serum deprivation. The incorporation of adenosine into adenine nucleotides may serve as an intracellular signal in the regulation of growth in mammalian cells.**

Low concentrations of adenosine have recently been shown to inhibit growth of various mammalian cells in culture (1–3). The growth-inhibitory properties of exogenous adenosine require the presence of adenosine kinase (EC 2.7.1.20) and were thus attributed to the formation of phosphorylated derivatives in mouse and hamster fibroblasts (1, 4). Toxicity of adenosine to lymphoid cells, however, is not dependent on the initial phosphorylation of adenosine (5). The addition of adenosine to cultures of mammalian cells can also cause effects similar to those resulting from the presence of exogenous cyclic AMP (6–9) which is known to possess growth-inhibitory properties (10). In several cases, extracellular catabolism of cyclic AMP by serum proteins yielded adenosine as the active ingredient in exogenous cyclic AMP action (7–9).

We now report that serum-deprived, growth-arrested cells of BALB/c 3T3 mouse (3T3), baby hamster kidney 21/13 (BHK), and simian virus 40-transformed BHK (SV-BHK) cell lines show an increase in the incorporation of exogenous adenosine into intracellular ADP and ATP pools compared to growing cells in serum-supplemented medium. The total ADP and ATP pool sizes are decreased after serum deprivation. Incubation with similar concentrations of inosine and hypoxanthine as well as adenine did not result in increased incorporation of the nucleoside or base precursor into the adenine nucleotide pools in serum-deprived cells. The decrease in the incorporation of uridine, cytidine, and guanosine into acid-soluble nucleotide pools after serum deprivation of mammalian cells is well documented (11, 12).

MATERIALS AND METHODS

All cell lines were free of mycoplasma contamination. Cells were periodically tested by the uridine/uracil incorporation procedure (13, 14) and the adenosine phosphorylase activity procedure (15).

Nearly confluent cultures of 3T3, simian virus 40-transformed 3T3 (SV-3T3), BHK, and SV-BHK cells in 25-cm^2 petri dishes were treated with Dulbecco's modified Eagle's minimal medium (DME) supplemented with 15% fetal calf serum and containing $^{32}P_i$ (10 μCi/ml) for 24 hr. Cultures containing the same number of cells were treated with DME without serum but containing $^{32}P_i$ for 24 hr. Cell counts at 0, 24, 32, and 40 hr after the medium change indicated that a 24-hr period in the presence of DME without serum resulted in effective arrest of growth of 3T3, BHK, and SV-BHK cells but not of SV-3T3 cells. Incorporation of [^{3}H]adenosine at 0.35 μM was performed after the 24-hr ^{32}P-labeling. [^{3}H]Adenosine incorporations were performed in DME without serum (1.5 ml per dish) containing the same ^{32}P specific activity as in the serum-supplemented and serum-deprived 24-hr media. Cells grown in serum-supplemented medium were washed twice with DME without serum containing the same specific activity of $^{32}P_i$. Cells to be incubated in DME without serum for 24 hr were washed twice with DME without serum after removal of the serum-supplemented medium.

After [^{3}H]adenosine incorporation, the medium was quickly removed, acid-soluble nucleotides were extracted with ice-cold 0.5 M perchloric acid, to which unlabeled carriers of ATP and ADP were added, for 45 min. The extracts were neutralized with 7 M KOH, the precipitate was removed by centrifugation, and the solution was lyophilized to dryness. Acid-soluble nucleotides (1 or 2% of the total) were chromatographed on PEI-cellulose thin-layer plates. The plates were developed with water and then with 1 M LiCl. The spots corresponding to the unlabeled carriers, visualized by their ultraviolet light absorption, were cut out, eluted with 4 M NH_4OH, and assayed for radioactivity in Bray's scintillation fluid. Total cellular ATP and ADP levels (pool sizes) were calculated by converting the ^{32}P cpm in the ATP and ADP fractions into nanomoles, on the basis of the specific activity of $^{32}P_i$ in the media. The ^{32}P specific activity was calculated from the phosphate concentration (determined for the two types of medium) and the ^{32}P radioactivity of the medium. The pool size determination was based on the assumption that the $^{32}P_i$ in the medium completely equilibrates with cellular $^{32}P_i$, [^{32}P]ATP, and [^{32}P]ADP within 24 hr, reaching similar specific activities. Experimental results have supported this assumption (16, 17). The ^{3}H specific activities of ATP and ADP were calculated from the ^{3}H and ^{32}P cpm. Analysis of ^{3}H-labeled nucleosides and bases in the [^{3}H]adenosine incorporation medium was performed on cellulose thin-layer plates developed with 1.8 M ammonium formate/2% boric acid neutralized to pH 7.0 or with *n*-butanol/

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Abbreviations: 3T3, BALB/c 3T3 mouse cell line; SV-3T3, simian virus 40-transformed 3T3 cell line; BHK, baby hamster kidney 21/13 cell line; SV-BHK, simian virus 40-transformed BHK; DME, Dulbecco's modified Eagle's minimal medium.

Table 1. Incorporation of [^{3}H]adenosine into ADP and ATP pools*

Incorporation time, min	Serum	[^{3}H]Adenosine specific activity, pmol/nmol [^{32}P]ATP or [^{32}P]ADP							
		3T3		SV-3T3		BHK		SV-BHK	
		ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP
0.5	+	0.28	0.35	0.21	0.30	0.39	0.73	0.58	1.25
	−	0.31	0.50	0.30	0.27	0.78	0.80	1.00	1.30
1	+	0.73	0.55	0.65	0.73	0.90	1.07	0.72	0.89
	−	1.08	0.94	0.64	0.58	1.35	1.68	1.50	1.83
5	+	1.70	1.79	1.87	2.15	2.50	1.82	1.17	1.48
	−	2.31	2.22	1.24	1.03	3.90	5.81	2.44	2.15
10	+	3.26	1.04	3.19	2.98	7.15	9.63	2.37	2.30
	−	5.56	4.75	2.42	2.55	10.55	11.73	3.95	4.41
Pool size†	+	5.4 ± 0.2	1.2 ± 0.1	5.2 ± 0.2	1.0 ± 0.2	10.1 ± 0.2	1.5 ± 0.1	9.8 ± 0.3	1.4 ± 0.2
	−	3.8 ± 0.4	0.7 ± 0.1	3.6 ± 0.4	0.5 ± 0.1	7.8 ± 0.5	1.2 ± 0.2	7.6 ± 0.2	1.1 ± 0.1

* Average of two experiments.
† Nanomoles per 10^6 cells, shown as mean ± SD for the four incorporation times.

propionic acid/water, 100:50:70 (vol/vol). Adenosine, adenine, inosine, and hypoxanthine were used as unlabeled markers.

RESULTS

The incorporation of [^{3}H]adenosine into ^{32}P-labeled ADP and ATP pools of serum-deprived cells and of cells growing in serum-supplemented medium is illustrated in Table 1. A decrease in the total intracellular ADP and ATP pool sizes occurred after 24 hr of serum deprivation. However, except in the case of SV-3T3, the incorporation of [^{3}H]adenosine into intracellular ADP and ATP pools was enhanced in the serum-deprived cultures compared to the growing, serum-supplemented cultures. The increase in adenosine incorporation into cellular ADP and ATP pools is related to the arrest of growth in G_1/G_0 phase of the cell cycle. This phenomenon did not occur in SV-3T3 cells which were not growth-arrested under the same serum-deprivation conditions, as was indicated by cell counts. Tables 2 and 3 show that the incorporation of precursors inosine and adenine into adenine nucleotide pools was not increased after 24-hr serum deprivation. After incubations with the four cell lines, thin-layer chromatographic analysis of the incorporation media showed conversion of [^{3}H]adenosine only to inosine and hypoxanthine. No formation of adenine was observed.

DISCUSSION

The unexpected increase in adenosine incorporation into growth-arrested cells as compared with growing cells may not have been noticed earlier because of the requirement for low concentrations of adenosine, similar to those present in human plasma (0.3–0.6 μM) (18). Higher concentrations of adenosine result in deamination of the majority of the adenosine to inosine by the action of cellular adenosine deaminase (EC 3.5.4.4). Adenosine concentrations have been shown to control adenosine metabolism because the K_m for adenosine in the adenosine kinase reaction (1.9×10^{-6} M) is well below the K_m for adenosine in the adenosine deaminase reaction (4×10^{-5} M) in human erythrocytes (19). Similar values have been obtained for other types of cells (20). Phosphorylation was shown to be the major pathway at concentrations approaching the K_m for adenosine kinase whereas higher concentrations of adenosine lead to a substantial increase in the deamination reaction (19, 21). The measurement of adenosine incorporation into cellular adenine nucleotides also requires the use of serum-free medium because the adenosine deaminase present in fetal calf serum (1, 22) leads to erroneous results.

The decrease in the incorporation of adenine, hypoxanthine, inosine, guanosine, cytidine, uridine, and thymidine into their corresponding nucleotide pools upon arrest of growth is expected as a result of the decrease in cellular demand for precursors of RNA and DNA synthesis. The increase in the incorporation of adenosine under the same conditions is unexpected and thus raises the possibility that the incorporation of adenosine into adenine nucleotides pools may act as a signal mediating the intracellular response to environmental changes that tend to slow cellular proliferation (23). Increases in intracellular adenine nucleotide pools, and in ATP in particular, have been associated with the transition from resting to growing states in various mammalian cells (17, 24). However, the expansion of the cellular ATP pool was shown to be stimulated by serum factor(s) that are different from the serum factor(s) that lead to initiation of DNA synthesis in quiescent cells (24). This, in turn, may suggest that the ATP pool size is not involved in the regulation of cellular proliferation.

The increases in adenosine incorporation into the adenine

Table 2. Incorporation of [^{3}H]inosine into ADP and ATP pools*

Incorporation time, min	Serum	[^{3}H]Inosine specific activity, pmol/nmol [^{32}P]ATP or [^{32}P]ADP							
		3T3		SV-3T3		BHK		SV-BHK	
		ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP
10	+	0.55	0.49	0.83	0.93	0.41	0.42	0.69	0.59
	−	0.48	0.36	0.35	0.30	0.23	0.21	0.39	0.33
Pool size†	+	6.3	1.4	5.9	1.1	9.5	1.9	9.2	1.7
	−	3.5	0.7	3.7	0.7	8.1	1.2	7.6	1.3

* All values are the average of two experiments. [^{3}H]Inosine incorporation medium was prepared by the addition of [^{3}H]adenosine (0.35 μM) to DME supplemented with 15% fetal calf serum. After 1-hr incubation at 37°, the [^{3}H]adenosine was converted to [^{3}H]inosine (80%) and [^{3}H]hypoxanthine (10–15%) as determined by cellulose thin-layer chromatography in two solvent systems. The medium was used at this stage.
† Nanomoles per 10^6 cells.

Table 3. Incorporation of [^{3}H]adenine into ADP and ATP pools*

Incorporation time, min	Serum	[^{3}H]Adenine specific activity, pmol/nmol [^{32}P]ATP or [^{32}P]ADP							
		3T3		SV-3T3		BHK		SV-BHK	
		ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP
1	+	0.76	1.72	0.65	0.76	0.17	0.26	0.14	0.32
	−	0.57	1.27	0.88	0.74	0.13	0.23	0.16	0.21
5	+	2.12	3.83	2.35	2.04	0.62	0.75	0.55	0.64
	−	0.66	1.33	2.18	2.53	0.62	0.52	0.64	0.77
10	+	2.80	3.59	3.80	3.42	1.05	1.36	1.21	1.60
	−	1.75	1.84	2.64	2.85	1.28	0.78	1.29	1.35
Pool size†	+	6.6 ± 0.6	1.6 ± 0.2	5.6 ± 0.3	1.2 ± 0.1	9.7 ± 1.2	2.0 ± 0.1	10.1 ± 0.9	2.0 ± 0.2
	−	3.9 ± 0.3	0.8 ± 0.1	3.5 ± 0.6	0.8 ± 0.3	7.7 ± 0.1	1.4 ± 0.2	8.2 ± 0.6	1.6 ± 0.2

* Average of two experiments. [^{3}H]Adenine incorporation medium included 0.65 μM [^{3}H]adenine (same concentration as in human plasma) (18) in DME without serum.

† Nanomoles per 10^6 cells, shown as mean ± SD for the three incorporation times.

nucleotide pools upon arrest of cellular proliferation is not simply a result of the decrease in the adenine nucleotide pool size. Recent data suggest that in Swiss 3T3 cells the expansion of the ATP pool in response to serum is due mainly to stimulation of *de novo* purine biosynthesis (24). The purine salvage pathways do not respond uniformly to serum deprivation. Whereas the incorporation of adenine, hypoxanthine, and inosine into the ATP and ADP pools is decreased in the quiescent state, the incorporation of adenosine is markedly increased.

Intracellular response to serum deprivation (negative pleiotypic response) (23) could possibly be regulated by formation of compartmentalized ATP produced exclusively from adenosine and not from adenine, hypoxanthine, or inosine. We have recently demonstrated that low levels of adenosine, but not adenine or hypoxanthine, lead to the formation of compartmentalized ATP in normal mouse liver *in vivo*. A rapidly proliferating human hepatoma grown subcutaneously in the *nude* mouse yielded much lower magnitudes of compartmentalized ATP from adenosine (22). Adenosine concentrations (0.1–0.5 mM) well above the level used in this study have been shown to expand the intracellular ATP pools in various cells (25–27). This effect could not be achieved with the other adenine nucleotides precursors (26).

The cells used in this study were mycoplasma-free; however, we have observed, along with others (13), that several lines of mammalian cells infected with adenosine phosphorylase-containing mycoplasmas (15) show an increase in their growth rates compared to uninfected cell lines. Whether the increase in the rate of cellular proliferation is related to the efficient degradation of intracellular adenosine to adenine by the adenosine phosphorylase activity remains to be established.

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