Homocysteine enhances the inhibitory effect of extracellular adenosine on the synthesis of proteins in isolated rat hepatocytes

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Previous work has shown that extracellular adenosine inhibits the incorporation of radiolabelled leucine into proteins in isolated rat hepatocytes [Tinton, Lefebvre, Cousin and Buc Calderon (1993) Biochim. Biophys. Acta **1176**, 1–6]. In this study, we investigated whether its metabolism into adenine nucleotides, inosine or *S*-adenosylhomocysteine (AdoHcy) is required to induce such an impairment. Incubation of isolated hepatocytes in the presence of adenosine at 0.5 or 1 mM reduces the synthesis of proteins by about 45% after 120 min of incubation. Such an inhibition occurred without cell lysis and was not modified by adding the adenosine kinase inhibitor 5-iodotubercidin (15 μM) or the adenosine deaminase inhibitor coformycin (0.1 μM). It is therefore unlikely that the anabolic and catabolic pathways of adenosine are involved in the inhibition of protein synthesis. Adenosine (1 mM) increased the level of AdoHcy and *S*-adenosylmethionine by 20- and 5-fold respectively after 60 min of incubation and reduced the methylation index. These events as well as the inhibition of protein synthesis were strongly enhanced in the presence of L-homocysteine (2 mM). It is therefore concluded that the metabolism of adenosine into AdoHcy, which is known to be a potent inhibitor of cellular methylation reactions, may play an important role in the control of translation.

INTRODUCTION

Adenosine can be released from cells that are metabolically active, exposed to stress conditions such as hypoxia, or damaged [1]. Once in the extracellular medium, adenosine can act as an efficient regulatory signal of many physiological processes by stimulating cell surface receptors (P_1 purinoceptors), coupled to the inhibition (A_1 and A_3 receptor subclasses) or the activation (A_2 receptor subclass) of adenylate cyclase [2].

We have previously reported that extracellular adenosine inhibits the synthesis of proteins in isolated rat hepatocytes [3]. Such an impairment was not prevented by P_1 receptor antagonists [4] and failed to correlate with an increase in free cytosolic calcium concentration [5], although this latter event has been reported to play an important role in the control of translation [6].

Besides its receptor-mediated effects, adenosine is rapidly taken up by mammalian cells [7] where it is metabolized by adenosine kinase (E.C.2.7.1.20), adenosine deaminase (E.C.3.5.4.4) and *S*-adenosylhomocysteine hydrolase (E.C.3.3.1.1) [8]. Several biochemical events, such as the formation of adenine nucleotides, the deficiency in adenosine deaminase or the accumulation of *S*-adenosylhomocysteine (AdoHcy), have been implicated in the adenosine-mediated toxicity towards many transformed or normal cell types of both human and animal origin [9]. In the present work, we investigated whether the metabolism of adenosine was involved in the inhibition of protein synthesis. Both 5-iodotubercidin, an inhibitor of adenosine kinase [10], and coformycin, an inhibitor of adenosine deaminase [11], were used to evaluate the involvement of the anabolic and catabolic pathways of adenosine in the impairment of protein synthesis. In addition, as we found that adenosine increased the levels of both AdoHcy and *S*-adenosylmethionine (AdoMet), we further investigated the effect of added L-homocysteine on the adenosine-mediated inhibition of protein synthesis.

MATERIALS AND METHODS

Chemicals

BSA (fraction V), adenosine, L-homocysteine thiolactone, 2-chloroadenosine and inosine were purchased from Sigma (St. Louis, MO, U.S.A). L-Homocysteine was prepared as described elsewhere [12] by cleavage of L-homocysteine thiolactone with 5 M NaOH. Collagenase was from Boehringer (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow Laboratories (Irvine, Ayrshire, U.K.). [U-^{14}C]Leucine and L-[^{35}S]methionine were purchased from the Radiochemical Centre (Amersham, U.K.). Coformycin was from Calbiochem (San Diego, CA, U.S.A.). 5-Iodotubercidin was from Research Biochemicals Incorporated (Natick, MA, U.S.A.). Rabbit reticulocyte lysate system for *in vitro* protein translation was from Promega (Madison, WI, U.S.A.). All other chemicals were of the purest grade available.

Preparation of hepatocytes

Hepatocytes were isolated and prepared as described previously [3] from male Wistar rats (250–280 g). The cells were resuspended in DMEM gassed with O_2/CO_2 (19:1) and supplemented with 0.3% BSA. The isolated hepatocytes (0.5×10^6 cells/ml) were shaken (100 cycles/min) in stoppered vials at 37 °C for 120 min, except where otherwise stated.

Assays

Hepatocyte viability was estimated by measuring the activity of lactate dehydrogenase (LDH) both in the culture medium and in the cell pellet obtained after centrifugation, as described elsewhere [13]. Results are expressed as the ratio of released activity to the total activity. Cell survival was calculated by subtracting the LDH leakage value from 100.

Abbreviations used: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; LDH, lactate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid.

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Protein synthesis was estimated by measuring the incorporation of [^{14}C]leucine (specific activity: 94 μCi/mmol, 0.8 mM unlabelled leucine) into the pelleted material obtained by perchloric acid precipitation as described before [14]. Results are expressed as d.p.m./mg of protein. The amount of proteins was determined by the Lowry method [15] using BSA as standard.

Total RNA was isolated by extraction with guanidinium thiocyanate followed by centrifugation in caesium chloride cushions, according to the standard procedure described elsewhere [16].

mRNA was separated from total RNA by affinity chromatography on oligo(dT)-cellulose and measured by absorbance at 260 nm. For translation studies *in vitro*, poly(A)$^+$ RNA (400 ng) was applied to a rabbit reticulocyte lysate system containing essential amino acids and [^{35}S]methionine (20 μCi). After different incubation times at 37 °C, samples were collected on filter papers and precipitated with ice-cold trichloroacetic acid (TCA) (10%, v/v). Nucleic acids were hydrolysed by boiling the filters in TCA (5%, v/v). The filters containing the residual protein pellets were then dried and counted. The results are expressed as d.p.m./2 μl of sample.

AdoHcy and AdoMet levels were assayed by HPLC on a cation-exchange column (supelcosil LC-SXC, Supelco, 250 × 4.6 mm). Briefly, 2×10^6 cells were collected and mixed with 100 μl of perchloric acid (5%). The samples were centrifuged as described in [17] and the acid-soluble supernatants were applied to the column and measured by using a binary elution programme. The chromatograph was a modular system from Spectra-Physics consisting of an SP-8800 solvent delivery system, an SP-8450 variable-wavelength detector and an SP-4290 detector–plotter. Solvent A was prepared by adding 10 mM $(NH_4)_2SO_4$ to 10% methanol in water and titrating to pH 2.9 with formic acid. Solvent B was prepared by adding $(NH_4)_2SO_4$ (100 mM) to solvent A. The elution programme (2 ml/min) started with 100% A and consisted of three linear gradients as follows: 1% B/min for 5 min, 2% B/min for 5 min and 5% B/min for 5 min. After completion of the run, the system was re-equilibrated for 7 min with 100% A before injection of the next sample. The column eluent was monitored at 258 nm and the results are expressed as pmol AdoHcy or AdoMet/10^6cells.

Statistics

Results are expressed as mean values ± standard error of the mean (S.E.M.) of at least three separate experiments. Analysis of variance (two-way ANOVA with the interaction time/treatment) was used to compare the concentration–response curves. For statistical comparison of results at a given time point, data were analysed using Student's *t*-test. A *P* value less than 0.05 was set as the minimum level of significance.

RESULTS AND DISCUSSION

Role of the catabolic and anabolic pathways of adenosine in the inhibition of protein synthesis

The reaction catalysed by adenosine deaminase leading to the formation of inosine represents the first step of the catabolic pathway of adenosine [8]. A significant inhibition of protein synthesis that reached 45% after 120 min of incubation was observed in hepatocytes treated with 0.5 mM adenosine (Table 1). Such an impairment was not modified by preincubating hepatocytes for 15 min in the presence of 0.1 μM coformycin which has been shown to inhibit adenosine deaminase in liver cells [11]. It has been reported that treatment of lymphoma cells of both human and murine origin with adenosine deaminase inhibitors leads to the impairment of protein, RNA and DNA synthesis [18–20]. As coformycin failed to prevent the inhibitory effect of adenosine, it is concluded that the catabolic pathway of adenosine was not involved in the inhibition of protein synthesis. This was further supported by the fact that inosine (0.5 mM) itself had no significant effect on the incorporation of radiolabelled leucine into proteins whereas 0.5 mM 2-chloroadenosine, an analogue resistant to adenosine deaminase [21], elicited an inhibition of protein synthesis which reached 60% after 120 min of incubation (Table 1).

Table 1 Role of the anabolic and catabolic pathways of adenosine in the inhibition of protein synthesis

Hepatocytes were preincubated for 15 min in the absence or in the presence of 0.1 μM coformicin (CF). Radiolabelled leucine was then added and cells were further treated for 120 min without or with adenosine (Ado), inosine, 2-chloroadenosine (2-ClAdo) and 5-iodotubercidin (Itu). The synthesis of proteins was estimated by measuring the incorporation of [^{14}C]leucine into proteins as described in the Materials and methods section. Results are means ± S.E.M of the number of experiments indicated in parentheses. * Student's *t*-test: significance level $P < 0.05$ as compared with control values.

Treatment	Protein synthesis (d.p.m./mg of protein)
Control	2356 ± 248 (4)
Ado (0.5 mM)	1482 ± 150 * (3)
CF (0.1 μM)	2290 ± 148 (3)
Ado (0.5 mM) + CF (0.1 μM)	1340 ± 195 * (3)
Itu (15 μM)	1455 ± 160 * (3)
Ado (0.5 mM) + Itu (15 μM)	1480 ± 280 * (3)
Inosine (0.5 mM)	2003 ± 268 (3)
2-ClAdo (0.5 mM)	728 ± 113 * (3)
CF (0.1 μM) + Itu (15 μM)	1342 ± 277 * (3)
Ado (0.5 mM) + CF (0.1 μM) + Itu (15 μM)	1384 ± 122 * (3)

We have previously reported that treatment of isolated hepatocytes with adenosine results in an important increase in cellular ATP level [4]. This leads us to investigate the involvement of the anabolic pathway of adenosine in the impairment of protein synthesis. When freshly isolated hepatocytes were incubated for 120 min in the presence of 15 μM 5-iodotubercidin, an inhibitor of adenosine kinase [10], the synthesis of proteins was reduced by 45% (Table 1). However, when adenosine and 5-iodotubercidin were added together, no further inhibition of protein synthesis occurred and the incorporation of radiolabelled leucine into proteins was reduced by 45% as compared with untreated cells. Such an impairment was similar to that induced by 0.5 mM adenosine alone.

Under basal conditions, the cytoplasmic 5′-nucleotidase of the liver cell continuously produces adenosine, which is immediately converted into AMP by adenosine kinase, without giving rise to allantoin [10]. The formation of AMP, resulting from the activity of adenosine kinase, has been reported to be an essential prerequisite for the inhibition of MCF-7 breast cancer cell proliferation [22]. When isolated hepatocytes were treated with 5-iodotubercidin the build-up of adenosine led to an increase in the level of AdoHcy as well as in the rate of formation of allantoin [10]. Since no additive effect on the inhibition of protein synthesis was observed by using adenosine and 5-iodotubercidin, our results suggest that a common mechanism, which does not require the phosphorylation of adenosine, was involved.

Accumulation of adenosine within the cells and its further metabolism by another biochemical pathway may thus play an important role in the inhibition of protein synthesis. Such a mechanism, once again, does not involve the catabolism of

Table 2 Effects of Hcy on the adenosine-mediated inhibition of protein synthesis

Hepatocytes were incubated for 120 min in the absence or in the presence of adenosine (Ado) and/or L-homocysteine (Hcy). Aliquots of cell suspension were taken at different times and the protein synthesis was estimated as described in the Materials and methods section. Values are means ± S.E.M. of the number of experiments indicated in parentheses. Student's *t*-test: significance level * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ as compared with control values.

Treatment	Protein synthesis rate (pmol Leu incorporated/min per mg of protein)
Control	74.5 ± 4.9 (9)
Hcy (2 mM)	54 ± 7.2* (9)
Ado (0.1 mM)	57.9 ± 6 (3)
Ado (0.1 mM) + Hcy (2 mM)	51.7 ± 10.9 (3)
Ado (0.25 mM)	46.6 ± 3.1* (3)
Ado (0.25 mM) + Hcy (2 mM)	35.9 ± 2.4** (3)
Ado (0.5 mM)	42.5 ± 1** (3)
Ado (0.5 mM) + Hcy (2 mM)	23.2 ± 3.3***(3)
Ado (1 mM)	42 ± 4.9** (3)
Ado (1 mM) + Hcy (2 mM)	4.9 ± 0.1*** (3)

adenosine. Indeed, as illustrated in Table 1, pretreatment of hepatocytes with coformycin modified neither the inhibitory effect induced by the combination of adenosine plus 5-iodotubercidin nor the inhibitory effect mediated by 5-iodotubercidin.

Role of the AdoHcy hydrolase pathway in the inhibition of protein synthesis induced by adenosine

S-Adenosylhomocysteine hydrolase catalyses the reversible hydrolysis of AdoHcy to adenosine and L-homocysteine. The equilibrium of the reaction favours the synthesis, but under physiological conditions the hydrolysis occurs due to the efficient removal of both adenosine and L-homocysteine by several enzymes [23]. Treatment of cultured or isolated cells as well as perfused organs with adenosine and/or L-homocysteine has been reported to increase the level of AdoHcy [9,23,24]. This metabolite, which is known to be a potent inhibitor of AdoMet-dependent transmethylation reactions of numerous molecules, such as DNA, RNA, proteins and phospholipids [23], has been implicated in the adenosine-mediated toxicity [9,23,25–27]. In T lymphoma cells deficient in adenosine kinase and treated with an inhibitor of adenosine deaminase, the adenosine-mediated cytotoxicity was greatly enhanced by addition of L-homocysteine thiolactone into the culture medium and was related to an accumulation of AdoHcy and to a further inhibition of DNA methylation [27]. Since no evidence of the role of the anabolic and catabolic pathways of adenosine was found, we investigated whether its condensation with L-homocysteine might be the key event leading to the inhibition of protein synthesis.

Incubation of isolated hepatocytes in the presence of 2 mM L-homocysteine decreased the rate of protein synthesis by 28% (Table 2). Adenosine also reduced the rate of protein synthesis, with a maximum occurring at 0.5 mM. Under these conditions, the incorporation of leucine into proteins was inhibited by 42% as compared with control cells. However, the simultaneous addition of 2 mM L-homocysteine reinforced the inhibitory effect of adenosine, which then became dependent on the concentration used. Indeed, compared with control hepatocytes, the rate of protein synthesis decreased by about 70% when cells were treated with 0.5 mM adenosine plus 2 mM L-homocysteine and by more than 90% when 1 mM adenosine and 2 mM L-homocysteine were used together. Neither 1 mM adenosine nor 2 mM L-homocysteine affected cell survival, as compared with control conditions (Figure 1a). A significant decrease in cell viability occurred when hepatocytes were incubated in the presence of both 1 mM adenosine and 2 mM L-homocysteine. Such an event, however, appeared only after 40 min of incubation and was unlikely to be involved in the inhibition of protein synthesis since, as compared with untreated cells, the incorporation of radiolabelled leucine into proteins was already reduced by 63% after 20 min of incubation (Figure 1b).

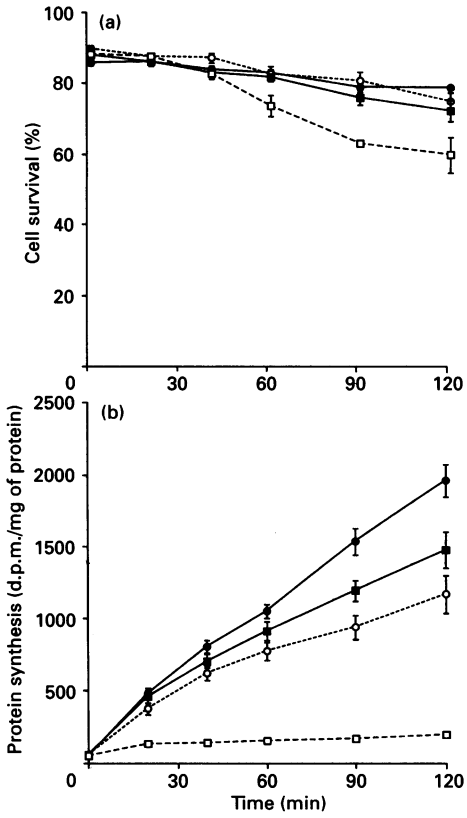

Figure 1 Effect of adenosine and L-homocysteine on cell survival and protein synthesis

Hepatocytes were incubated for 120 min in the absence (●) or in the presence of 1 mM adenosine (○, broken line), 2 mM L-homocysteine (■) or a combination of both adenosine plus L-homocysteine (□, broken line). At the times indicated, cell survival (**a**) and protein synthesis (**b**) were measured as described in the Materials and methods section. Results are means ± S.E.M. of 3–9 separate experiments.

Our results therefore indicated that the saturating inhibitory effect of adenosine at concentrations of 0.5 and 1 mM probably resulted from the limiting availability of cellular L-homocysteine. As exogeneous L-homocysteine potentiated the inhibition of protein synthesis induced by adenosine, the formation of AdoHcy catalysed by *S*-adenosylhomocysteine hydrolase may be an important event leading to the impairment of protein synthesis. Indeed, 1 mM adenosine and 2 mM L-homocysteine, which produced significant inhibition of protein synthesis, increased by about 22- and 34-fold respectively the level of AdoHcy as compared with control hepatocytes (Table 3). These compounds also raised by 5-fold the content of AdoMet after 60 min of incubation and reduced significantly the methylation index. However, a stronger effect on both the AdoHcy and AdoMet levels was observed when these compounds were added together

Table 3 Effect of adenosine and L-homocysteine on cellular levels of AdoHcy and AdoMet

Hepatocytes (2×10^6 cells/ml) were incubated for 60 min in the absence or in the presence of adenosine (Ado, 1 mM), L-homocysteine (Hcy, 2 mM) or a combination of both Ado plus Hcy. The cellular levels of AdoHcy and AdoMet were measured as described in the Materials and methods section. Results are expressed as pmol/10^6 cells. The methylation index was evaluated by calculating the AdoMet/AdoHcy ratio. Values are means ± S.E.M. of three separate experiments. * Student's *t*-test: significance level $P < 0.05$ as compared with control values.

	AdoHcy (pmol/10^6 cells)	AdoMet (pmol/10^6 cells)	Methylation index
Control	96 ± 14	250 ± 52	2.77 ± 0.66
Ado (1 mM)	2072 ± 304*	1308 ± 336*	0.61 ± 0.07*
Hcy (2 mM)	3272 ± 91*	1424 ± 98*	0.44 ± 0.13*
Ado (1 mM) + Hcy (2 mM)	17301 ± 2038*	3451 ± 620*	0.20 ± 0.02*

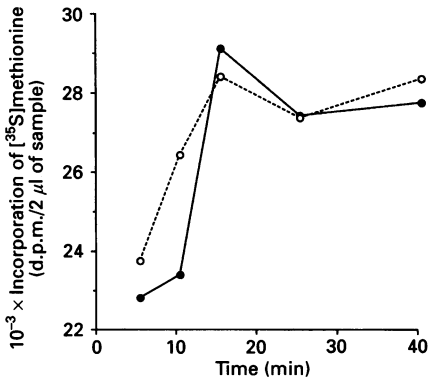

Figure 2 Effect of adenosine on the translation of mRNA into proteins

Hepatocytes were incubated for 120 min in the absence (●) or in the presence of 1 mM adenosine (○, broken line). Cells were then collected and the mRNA was prepared as described in the Materials and methods section and applied to a translation system *in vitro*. The ability of mRNA to be translated was evaluated at different times by measuring the incorporation of [^{35}S]methionine into proteins. Results are expressed as d.p.m./2 μl of sample.

to the cell suspension. Under these conditions the content of AdoHcy was 180-fold higher than that measured in untreated cells, whereas the AdoMet increased by about 14-fold. The methylation index was also further reduced as it represented only 5% of the control value.

Effect of adenosine on the translation of mRNA into proteins

As hypomethylated molecules may be involved in the impairment of protein synthesis, we investigated the effect of adenosine on the ability of mRNA to be translated into proteins. As illustrated in Figure 2, the mRNA of hepatocytes incubated for 120 min in the presence of 1 mM adenosine was translated into proteins at the same rate as the mRNA of control hepatocytes. In addition, the inhibitory effect on the synthesis of proteins was probably not related to a degradation of total RNA, since the electrophoretic profile obtained when cells were treated for 120 min with 1 mM adenosine was similar to that observed with untreated cells (results not shown).

In conclusion, both the inhibition of protein synthesis and the accumulation of AdoHcy induced by adenosine were strongly enhanced by L-homocysteine. This suggests that the pathway of *S*-adenosylhomocysteine hydrolase plays an important role in the control of translation. AdoHcy has been reported to be a potent inhibitor of AdoMet-dependent transmethylation reactions of nucleic acids and proteins. It is however unlikely that hypomethylation of mRNA was involved in the adenosine-mediated inhibition of protein synthesis, since mRNA kept its ability to be translated into proteins. At present, the nature of the hypomethylated molecule(s) being responsible for the adenosine-mediated protein synthesis inhibition is(are) unknown. The hypomethylation of ribosomal RNA leading to a defect in protein synthesis has been reported in hepatocytes treated with several hepatotoxins such as CCl_4, ethionine and D-galactosamine [28]. Although D-galactosamine decreased the level of ATP and AdoMet whereas adenosine produced opposite effects, we cannot exclude that hypomethylation of ribosomal RNA may also play an important role in the inhibition of protein synthesis observed under our experimental conditions. Such an hypothesis needs to be further investigated.

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