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Changes in polyamine content in primary cultures of adult rat hepatocytes

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Primary cultures of rat hepatocytes are a useful model system for the study of drug metabolism and toxicity [1, 2]. These are non-dividing cells which, when maintained under conventional culture conditions, exhibit rapid phenotypic changes resulting in a marked decrease in highly differentiated functions such as their ability to metabolise drugs via the cytochrome P-450 mono-oxygenase system [3, 4].

The polyamines, spermidine and spermine, and their diamine precursor, putrescine, are naturally occurring cellular polycations found in all living cells [5]. Their precise function within the cell is not known but increasing evidence indicates they play a positive role in the regulation of cell growth [6, 7].

Recently, it has been shown that polyamines have differential effects on the functions of freshly isolated hepatocytes. For example, polyamines can alter the cAMP-mediated stimulation of amino acid transport [8] and can inhibit the hormonal induction of tyrosine aminotransferase [9]. No study has, however, examined the changes in polyamine metabolism in primary cultures of hepatocytes. In this paper we have measured the alterations in the polyamine content of rat hepatocytes with time in primary culture and the effect inhibitors of polyamine metabolism have on these changes.

Materials and methods

Waymouth's medium was purchased from Flow Laboratories Ltd (Rickmansworth, U.K.). Donor horse serum, penicillin and streptomycin were from Gibco-BRL Ltd. Polyamines, hydrocortisone-21-acetate, insulin and inhibitors were from Sigma Chemical Co. (Poole, Dorset, U.K.). Collagenase was from Boehringer (Mannheim, F.R.G.).

Hepatocytes were isolated from male Sprague–Dawley rats (180–280 g) under aseptic conditions [10] and cultured on 10 cm dia. Petri dishes in Waymouth's medium supplemented with 1% (v/v) horse serum 10 μM-hydrocortisone and 1 μM insulin. Viability was assessed by Trypan blue exclusion and was initially 92% or greater. Polyamines were extracted from the cells in 0.2 M $HClO_4$ as described previously [11] and quantified by HPLC [12]. Protein content was determined by the method of Lowry *et al.* [13]. Each experiment is representative of a number carried out under the same conditions. In each case a range of the values obtained within one experiment is given. There was inter-rat variability in terms of the polyamine content but the results were consistent within each experiment. MGBG was extracted and quantified by HPLC [14].

Results and discussion

In rapidly growing cells the polyamine content on the cells increases with the cell growth rate [15]. In non-transformed cells, such as BHK-21/C13 cells, spermidine is the major polyamine within the cells and its intracellular concentration rises while the cells continue to grow. Primary cultures of hepatocytes do not grow in tissue culture but rather are maintained in a viable condition for several weeks [16]. As non-growing cells they therefore provide a novel system in which to study the role of polyamines. Initial experiments showed that the total polyamine content in freshly isolated hepatocytes was high and that this value decreased rapidly up to 24 hr. The total polyamine content of these cells remained relatively constant thereafter (Fig. 1). A more detailed analysis of the early times in culture showed that the total polyamine content remained approximately constant for 6–7 hr in culture; thereafter it decreased with time (Table 1). During this time the distribution of the individual polyamines was changing as seen by the continuous decline in the ratio of spermidine to spermine (Table 1). This ratio is a useful index of cell growth rate in non-transformed cells, high ratios of 1.5–2.0 being indicative of rapid rates of cell growth. This ratio falls markedly when cell growth slows down and, at confluence can be as low as 0.25 [15]. The low ratio in hepatocytes, even at early times, is indicative of their non-growing state.

Analysis of the individual polyamines showed spermine as the major polyamine within hepatocytes (Fig. 2). Putrescine, the precursor of spermidine and spermine was not detected at early times. Both spermidine and spermine decreased with time in culture, the former decreasing more quickly and hence accounting for the decline in the ratio of these two polyamines. Despite the fall in the content of the higher polyamines the total polyamine content was maintained, at least initially. This was due to the production of both putrescine and N^1-acetylspermidine from 3 hr onwards (Fig. 1). N^1-acetylspermidine is formed by the action of spermidine/spermine *N*-acetyltransferase which is the rate-limiting reaction in the breakdown or "so-called" retroconversion of the higher polyamines [17]. Putrescine

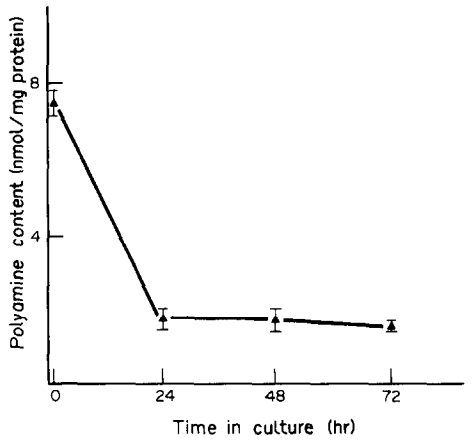

Fig. 1. Polyamine content of primary cultures of adult rat hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats as described in Materials and Methods. Cell monolayers were washed twice with ice-cold phosphate buffered saline and the cells harvested mechanically. Polyamines were extracted in 0.2 M $HClO_4$ and analysed by HPLC [11, 12]. Values are the mean ± SD (N = 4).

Table 1. Total polyamine content of primary cultures of adult rat hepatocytes

Time in culture (hr)	Total polyamine content (nmoles/mg protein)	Spermidine Spermine
1	7.36	0.79
2	7.84	0.78
3	8.10	0.76
4	7.31	0.65
5	6.52	0.61
6	7.53	0.54
7	5.83	0.42
8	6.86	0.40
9	5.61	0.35
19	3.51	0.24
24	3.03	0.32

Polyamines were extracted as described previously [11] and analysed by HPLC [12]. Values are the average of two analyses. The range was less than 10%.

is also a product of this catabolic pathway being formed from N^1-acetylspermidine under the action of polyamine oxidase [17]. Putrescine can also be formed from ornithine via ornithine decarboxylase (ODC)* the rate-limiting enzyme in polyamine biosynthesis [18]. ODC is a highly responsive enzyme which is readily induced by a variety of stimuli usually, however, stimuli which increase the growth rate of the cell [5]. In order to confirm that biosynthesis was not a major pathway in these cells we cultured the hepatocytes in the presence of 10 μM-MGBG, a known inhibitor of polyamine biosynthesis [19]. This concentration of MGBG has been shown to be cytostatic to BHK-21/C13 cells growing in monolayer culture [20]. MGBG had no effect on the total polyamine content despite a significant concentration of MGBG within the cells (Table 2). This intracellular MGBG concentration represents a 200–300-fold concentration gradient across the cell membrane, and is a concentration sufficient to prevent biosynthesis of polyamines in a resistant human cancer cell line [21]. This result combined with the loss of both spermidine and spermine and the formation of putrescine and N^1-acetylspermidine would suggest that biosynthesis was not taking place in these cells to any appreciable extent but that retroconversion was occurring. The reason for the breakdown of the higher polyamines is unclear. It may be that the hepatocytes contain too high a positive charge and breakdown of spermidine and spermine decreases this. In the light of previous work, in quiescent cells the most likely reason for the breakdown is that these non-growing cells do not require the "growth promoting" properties of the

* Abbreviations used: ODC, ornithine decarboxylase; MGBG, methylglyoxal bis(guanylhydrazone).

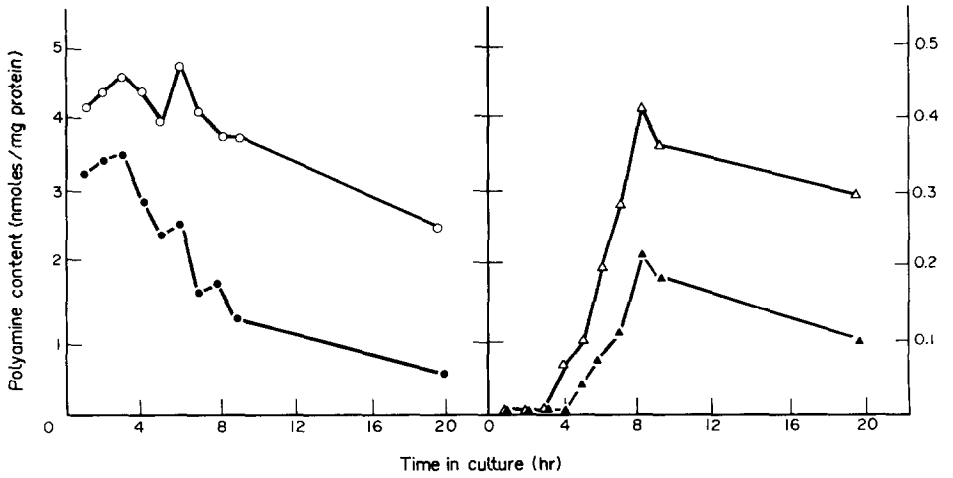

Fig. 2. Distribution of polyamines in primary culture of adult rat hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats as described in Materials and Methods. Cell monolayers were washed twice with ice-cold phosphate buffered saline and the cells harvested mechanically. Polyamines were extracted in 0.2 M $HClO_4$ and analysed by HPLC [11, 12]. (a) ○——○, spermine; ●——●, spermidine; (b) △——△, putrescine; ▲——▲, N^1-acetylspermidine. Values are the average of duplicate analyses. The range was ± 9%.

Table 2. Effect of MGBG on polyamine content of primary cultures of adult rat hepatocytes

Time in culture (hr)	Additions	MGBG content (nmol/mg of protein)	Total polyamine content (nmol/mg of protein)
24	none	n.d.	2.38 ± 0.41
	10 μM-MGBG	4.37 ± 0.28	2.30 ± 0.32
48	none	n.d.	2.07 ± 0.22
	10 μM-MGBG	12.49 ± 1.46	2.16 ± 0.10

Hepatocytes were cultured in the presence or absence of 10 μM-MGBG. MGBG was dissolved in 0.9% (w/v) NaCl and filter-sterilised before use. Values are the mean ± SEM (N = 3). n.d. not detected.

MGBG and polyamines were extracted and measured by HPLC [12, 14].

polyamines therefore the cell catabolises the higher polyamines as a prerequisite to their excretion. This is directly analogous to confluent (i.e. non-growing) cultures of BHK-21/C13 cells where polyamine excretion has been shown to be a major mechanism whereby the cell can dispose of excess amines [15].

Primary cultures of hepatocytes therefore provide a unique system in which to study polyamine catabolism in mammalian cells.

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