Uptake of polyamines by human endothelial cells

Characterization and lack of effect of agonists of endothelial function

David M. L. MORGAN

Vascular Biology Research Centre, Biomedical Sciences Division, King's College London, Campden Hill Road, London W8 7AH, U.K.

Uptake of polyamines by confluent monolayers of human umbilical-vein endothelial cells (HUVECs) was found to be time-, temperature- and concentration-dependent, energy-requiring, and saturable. Kinetic constants were putrescine K_t $3 \pm 1 \mu\text{M}$, V_{max} $15 \pm 7 \text{ pmol/h per } \mu\text{g}$ of protein; spermidine, 0.7 ± 0.2 , 12 ± 3 ; spermine, 1 ± 0.7 , 11 ± 4 . Putrescine uptake was inhibited by spermine or spermidine, whereas uptake of spermine or spermidine was not inhibited by $20 \mu\text{M}$ -putrescine. These data suggest the existence of two carriers, one shared by spermine and spermidine, and one capable of transporting all three polyamines. Pretreatment of HUVECs with thrombin ($\leq 10 \text{ units/ml}$; 1 h), bradykinin ($\leq 10 \mu\text{M}$; 1 h), interleukin-1 ($\leq 100 \text{ units/ml}$; 2 h) or phorbol 12-myristate 13-acetate ($\leq 1.0 \mu\text{M}$; 1 h), all known agonists of endothelial function, had no significant effect on polyamine uptake. These responses may be of importance in angiogenesis and wound healing, and could have pharmacological significance, for there is a growing interest in the use of polyamines or polyamine analogues as therapeutic agents.

INTRODUCTION

The polyamines putrescine ($\text{NH}_2[\text{CH}_2]_4\text{NH}_2$), spermidine ($\text{NH}_2[\text{CH}_2]_6\text{NH}[\text{CH}_2]_4\text{NH}_2$) and spermine ($\text{NH}_2[\text{CH}_2]_8\text{NH}[\text{CH}_2]_3\text{NH}[\text{CH}_2]_4\text{NH}_2$) are normal cellular constituents essential for growth and differentiation [1–4], and references therein). Their intracellular concentrations vary with transit through the cell cycle, and induction of ornithine decarboxylase, a rate-limiting enzyme in polyamine biosynthesis, is a very early event in cell proliferation. Both platelets and red cells, though non-dividing, are rich in polyamines, which are released during platelet activation or haemolysis [5,6]. Polyamines can modulate agonist-induced platelet activation [7–9], and when oxidized by plasma polyamine oxidases become highly cytotoxic [10,11].

The vascular endothelium, once thought to be merely a passive lining which acted as a filter between the blood and the vessel wall, is now known to contribute to the maintenance of vascular homeostasis and a range of endothelial-cell functions have been characterized at the molecular level [12–14]. Polyamines, at low concentrations, enhance the growth rate of human umbilical-vein endothelial cells (HUVECs) in culture [10], suggesting that these cells take up polyamines. I have now characterized the uptake of polyamines by monolayers of HUVEC and found it to be time-, temperature- and concentration-dependent, energy-requiring, and saturable. The presence in vascular endothelial cells of a transport system, capable of accumulating polyamines and perhaps polyamine analogues, may be of importance in angiogenesis and could have pharmacological significance, for there is a growing interest in the use of polyamine analogues as therapeutic agents [3,15].

EXPERIMENTAL

Materials

Spermine, spermidine, putrescine, N^1 -acetylspermidine, N^8 -acetylspermidine, N^1 -acetylspermine, N^1 -acetylputrescine, L-

lysyl-L-lysine (as the hydrochlorides) and BSA were from Sigma Chemical Co., Poole, Dorset, U.K. N^1N^{12} -Diacetylspermine was synthesized as described previously [10]. Difluoromethylornithine was a gift from Marion Merrell Dow, Cincinnati, OH, U.S.A.; calf serum was from Globepharm, Esher, Surrey, U.K. Tissue-culture media (in powder form) were from Gibco, Paisley, Scotland, U.K. Bradford protein reagent was from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K. [^3H]Putrescine {[1,4(n)- ^3H]tetramethylenediamine dihydrochloride (22.9 Ci/mmol)}, [^{14}C]spermine { NN' -bis-(3-aminopropyl)-[1,4- ^{14}C]tetramethylene-1,4-diamine tetrahydrochloride (118 mCi/mmol)} and D-[1- ^{14}C]mannitol (50 mCi/mmol) were from Amersham International, Amersham, Bucks., U.K. [^3H]Spermidine { N -(3- ^3H)aminopropyl-1,4- ^3H]diamine trihydrochloride (50.6 Ci/mmol)} was from DuPont-NEN, Stevenage, Herts., U.K. Scintiran-T scintillation fluid was from BDH, Poole, Dorset, U.K.

Cell cultures

HUVECs were isolated from umbilical cords as described elsewhere [10,16]. Confluent second-passage cells were detached by brief treatment with trypsin (0.1%) plus EDTA (0.02%) in phosphate-buffered saline (PBS; PBS 'A' [17]) and resuspended ($5 \times 10^4 \text{ cells/ml}$) in growth medium [Medium 199 supplemented with 10% (v/v) foetal-calf serum, 10% (v/v) newborn-calf serum, 2 mM-glutamine, penicillin and streptomycin (each 100 I.U./ml)]. Sub-samples (200 μl ; approx. 10^4 cells) were dispensed into 60 wells of a flat-bottomed micro-test plate [10], and allowed to adhere for 24 h before use. When cells were treated with difluoromethylornithine, this was dissolved in water, adjusted to pH 7.4 with 1 M-NaOH and added to growth medium to a final concentration of 5 mM 48 h before the commencement of the experiment.

Measurement of polyamine uptake

Medium was removed from each well and the cells were rinsed with a buffered salt solution (MHBSS; Hanks balanced salt solution [18] modified by addition of 20 mM-Hepes and adjusted to pH 7.4 by addition of 5 M-NaOH), or the medium to be used

in that experiment. All operations were carried out at 37 °C. Radiolabelled polyamine (diluted to 1 mCi/mmol with unlabelled material; [^{14}C]spermine was used as supplied) was added in 50 μl of MHBSS. After incubation for 15 min, or other indicated time, when < 2% of the applied label had been taken up, the plates were placed in melting ice and 100 μl of ice-cold PBS was added immediately. Cell monolayers were then rinsed three times with 200 μl portions of ice-cold PBS (addition of 1 mM unlabelled polyamine to the wash did not affect the results, and so this was omitted). When cells were pretreated, the agent, dissolved in MHBSS, was added for the specified time, then removed before the addition of polyamine. In cross-inhibition studies the competing polyamine was added at the same time as the radiolabelled substrate. Na^+ -dependence was determined by using a modified Krebs–Henseleit salt solution as control (140 mM-NaCl, 5.6 mM-KCl, 2.5 mM- CaCl_2 , 1.0 mM- MgCl_2 , 2.5 mM- NaHCO_3 , 1.0 mM- Na_2HPO_4 , 5 mM-glucose, 20 mM-Hepes, pH 7.4), with progressive replacement of Na^+ by choline in experimental media. Treatments were replicated in ten wells in each experiment, and each experiment was repeated at least three times (for kinetic experiments $n = 6$).

Measurement of cell protein

The protein content of each well was determined after addition of 100 μl of Coomassie Blue protein reagent [19], diluted 1:10 before use. BSA standards and blanks were placed in the unused outer wells of each plate. After incubation for 30 min at room temperature, A_{620} was measured in a Multiskan plate reader (Flow Laboratories, Irvine, Scotland, U.K.).

Measurement of radiolabel uptake

Formic acid (25 M; 100 μl /well) was added in the presence of the protein reagent. After 30 min the contents of each well were transferred, with one rinse of PBS, to a polyethylene mini-vial and 3.5 ml of Scinttran-T scintillation fluid was added. Radioactivity was determined in a Wallac/Rackbeta liquid-scintillation counter. Portions (10 μl) of each test solution were included in each batch of samples counted for radioactivity. Background radioactivity was determined by including samples from which only the radiolabel was omitted.

Data analysis

Apparent kinetic constants (K_t , V_{max} , K_i) were estimated by computer fitting of the experimental data to Michaelis–Menten or Dixon plots. Where appropriate, data were analysed by a multiple means comparison test [20] with the overall confidence level set at 99% ($P > 0.01$ was considered not significant).

RESULTS

Removal of non-cell-associated radiolabel was examined by the inclusion of a radiolabelled extracellular marker, [^{14}C]mannitol, in some experiments. As shown (Fig. 1b), negligible amounts of marker remained trapped in the extracellular space at the end of the washing procedure. Uptake of radiolabel was much decreased in the presence of excess unlabelled substrate (Fig. 1a) and also at low temperature (Fig. 1c). Uptake of radiolabelled polyamine by HUVECs was linear with time up to 20 min (results not shown); hence experiments were carried out at 15 min unless otherwise noted.

Apparent kinetic constants for the uptake of putrescine, spermidine and spermine, determined at 37 °C with 0.25–10 μM

polyamine, are given in Table 1. Competition between the polyamines for the carrier(s) was examined by adding increasing concentrations (1–20 μM) of the competing polyamine in the presence of a fixed concentration (5 μM) of the substrate polyamine. Putrescine uptake was inhibited by spermine or spermidine, whereas uptake of spermine or spermidine was not inhibited by putrescine at concentrations up to 20 μM . Spermine inhibited spermidine uptake, and vice versa (Table 2).

Fig. 1. Polyamine uptake by HUVECs in culture

Effect of adding 10 mM unlabelled putrescine (●) on the uptake of [^3H]putrescine (○). The line indicated by ■ was obtained by subtraction. (b) Cell-associated radioactivity derived from [^3H]spermidine (○) and the extracellular marker [^{14}C]mannitol (●) added at 15000 d.p.m./well. (c) Spermine uptake at 37 °C (○) and 0 °C (●). The line indicated by ■ was obtained by subtraction. Error bars show S.D. ($n = 8$ –10).

Table 1. Kinetic constants for polyamine uptake by HUVECs

Uptake was measured at 15 min at concentrations of 0.25–10 μM . Results are expressed as means $\pm$ S.D., $n = 6$ experiments (see the Experimental section).

	K_t (μM)	V_{max} (pmol/h per μg of protein)
Putrescine	3 ± 1	15 ± 7
Spermidine	0.7 ± 0.2	12 ± 3
Spermine	1 ± 0.7	11 ± 4

Table 2. Inhibitory effects of polyamines on polyamine uptake by HUVECs

Uptake was measured at 15 min with 5 μM substrate and inhibitor concentrations of 1–20 μM . Results are expressed as means $\pm$ S.D., $n = 3$ experiments.

Substrate	Inhibitor	K_i (μM)
Putrescine	Spermidine	0.6 ± 0.2
	Spermine	0.4 ± 0.1
Spermidine	Spermine	1 ± 0.3
Spermine	Spermidine	1 ± 0.7

Table 3. Effects of *N*-ethylmaleimide, 2,4-dinitrophenol, *p*-chloromercuribenzoate or dithiothreitol on polyamine uptake by HUVECs in culture

Polyamine concentrations were 5 μM . Cells were exposed to the agent for 20 min, which was then removed before addition of the polyamine. Values are percentages of control uptake, means $\pm$ S.D. ($n = 3$ experiments). All results are significantly different from control values ($P < 0.01$). Uptake of putrescine and spermidine in control cultures was 5.3 ± 0.9 and 7.7 ± 0.5 pmol/h per μg of protein respectively.

Agent	Uptake (%)	
	Putrescine	Spermidine
None	100 ± 16	100 ± 11
<i>N</i> -Ethylmaleimide (5 mM)	13 ± 3	34 ± 8
<i>p</i> -Chloromercuribenzoate (50 μM)	19 ± 5	35 ± 7
Dinitrophenol (2 mM)	14 ± 1	28 ± 5
Dithiothreitol (10 mM)	141 ± 16	215 ± 13

Fig. 2. Effects of alterations in the extracellular Na^+ concentration on the uptake of putrescine (○), spermidine (●) and spermine (■) by HUVECs in culture (polyamine concn. 5 μM)

Error bars show S.D., $n = 8$ –10 observations. For details see the Experimental section.

Preincubation with lysyl-L-lysine (M_r 347; 2.5–20 μM) for 15 min or concomitant addition of the peptide did not inhibit polyamine uptake (results not shown). Co-incubation with acetyl-polyamines at concentrations up to 20 μM was not inhibitory. Thus N^1 -acetylputrescine did not inhibit putrescine uptake, and spermidine uptake was not inhibited by N^1 -acetylspermidine or by N^8 -acetylspermidine; nor was spermine uptake inhibited by N^1 -acetylspermine or N^1N^{12} -diacetylspermine (results not shown). Prior exposure of cells to *N*-ethylmaleimide (5 mM), *p*-

chloromercuribenzoate (50 μM) or 2,4-dinitrophenol (2 mM) for 20 min inhibited polyamine uptake by $> 70\%$ (Table 3). In contrast dithiothreitol (10 mM) enhanced uptake by 50–100%.

Putrescine and spermidine uptake decreased by 70% when the Na^+ content of the medium was decreased (Fig. 2), whereas spermine uptake was not affected. Exposure to difluoromethylornithine (5 mM, 48 h), an irreversible inhibitor of ornithine decarboxylase, resulted in an increase in uptake of putrescine or spermidine from 2.6 ± 0.4 or 1.8 ± 0.3 to 6.4 ± 0.1 or 4.9 ± 0.4 pmol/h per μg of protein respectively (polyamine concn. 5 μM ; $n = 3$). Pretreatment of endothelial cells with thrombin (1–10 units/ml; 1 h), bradykinin (1–10 μM ; 1 h), interleukin-1 (1–100 units/ml; 2 h) or phorbol 12-myristate 13-acetate (0.1–1 μM ; 1 h) had no significant effect on polyamine uptake (results not shown).

DISCUSSION

Polyamine uptake by HUVECs was found to be time-, temperature- and concentration-dependent and to be saturable (Fig. 1). Kinetic constants for polyamine uptake (Table 1), when compared with polyamine levels in normal human plasma (putrescine, 0.1–0.2 μM ; spermidine, 0.1–0.3 μM ; spermine 0.03–0.04 μM ; [21], and references therein), indicate that under normal circumstances endothelial polyamine carriers are unlikely to be saturated and therefore capable of clearing locally generated polyamines (resulting from, e.g., haemolysis, injury etc.) Intracellular polyamine concentrations are, in general, 10–100-fold greater than extracellular levels [21], hence polyamine accumulation would appear to proceed against a substantial concentration gradient. However, it is likely that only the intracellular 'free' polyamine pool is of importance in the regulation of uptake, and work is needed to investigate this point.

The inhibition of putrescine uptake by spermine or spermidine, and the lack of effect of putrescine on the uptake of spermine or spermidine, suggest the existence of two carriers, one shared by spermine and spermidine, and one capable of transporting all three polyamines. A number of cell types are known to possess a polyamine uptake system which, under suitable conditions, can substitute for synthesis *de novo* [22,23]. In general the properties of these systems are not well defined [24–27], and only in recent reports are there suggestions of the number of carriers involved [28–33].

Byers & Pegg [34] proposed the existence of two adjacent polyamine-transport recognition sites on Chinese-hamster ovary cells, one with affinity for terminal aminobutyl groups and the other directed towards terminal aminopropyl moieties. The two putative carriers in endothelial cells suggested by the data in Table 2 could have similar properties. However, spermidine and putrescine transport were both partially Na^+ -dependent, whereas spermine uptake was Na^+ -independent (Fig. 2). Putrescine uptake in a number of cell types has been reported to be at least partially Na^+ -dependent [35–38]. The data relating to spermidine uptake are more variable, Na^+ -dependence having been observed in pneumocytes [37], enterocytes [30] and pulmonary epithelial cells [39], but not in adrenocortical or B16 melanoma cells [33,35]. Spermine uptake is generally Na^+ -independent [35,37]. Partial Na^+ -dependency has been interpreted as indicating the presence of two transporters, one Na^+ -dependent, the other Na^+ -independent [40,41]. An alternative explanation is that the putative spermidine/putrescine carrier in endothelial cells is activated by Na^+ rather than being Na^+ -dependent. This is supported by the residual 30% uptake observed in the absence of Na^+ (Fig. 2). These results are difficult to reconcile with the two-carrier model proposed above, though it is possible to argue that spermine (in the presence of Na^+) blocks, but it is not transported by, a Na^+ -

dependent carrier for putrescine and spermidine. The existence of multiple carriers with overlapping specificities has been proposed previously [32,34], and the ability of a single carrier to transport more than one polyamine was confirmed by Byers *et al.* [42], who by transfection of a single human gene induced transport of putrescine and spermine in a polyamine-transport-deficient mutant Chinese-hamster ovary cell line.

Lysyl-L-lysine, a molecule comparable in size and charge with spermidine or spermine, did not inhibit polyamine uptake. Hence molecular size and charge alone are insufficient to ensure binding to the transporter, and some degree of stereospecificity must be involved.

Inhibition of ornithine decarboxylase, a rate-limiting enzyme in polyamine biosynthesis, which is known to decrease intracellular putrescine and spermidine [2,3], increased the uptake of both putrescine and spermidine, but not spermine, by endothelial cells. Similar findings have been reported for many other cell types [34,40,43]. These data suggest that one result of intracellular polyamine depletion may be induction of the polyamine transporter(s).

Prior exposure of endothelial cells to *N*-ethylmaleimide or *p*-chloromercuribenzoate, reagents that alkylate thiol groups (the former can be taken up by cells, whereas the latter is relatively impermeant and hence more likely to react with groups on the cell surface), resulted in a marked inhibition of polyamine uptake (Table 3). In contrast, dithiothreitol, a thiol-group donor, enhanced uptake, indicating that in these, as in other cell types, polyamine transport is facilitated by the presence of thiol groups [29,40,44,45]. 2,4-Dinitrophenol (an uncoupler of mitochondrial oxidative phosphorylation) also exerted a profoundly inhibitory effect, demonstrating that polyamine uptake by these cells is an energy-requiring process, a conclusion supported by the decrease in uptake at low temperature (Fig. 1).

Endothelial-cell secretory activities can be categorized as being of two types: either rapid and transient (minutes) or sustained (hours), the latter being slower in onset. Thrombin and bradykinin each rapidly stimulate transient prostacyclin release, and bradykinin also stimulates the rapid synthesis and expression of platelet-activating factor by these cells, whereas phorbol 12-myristate 3-acetate or thrombin induce the acute secretion of von Willebrand factor, a high-molecular-mass glycoprotein stored in secretory granules. In addition to these rapid responses, the phorbol ester chronically elevates constitutive release of von Willebrand factor, and induces long-term secretion of collagenase. Interleukin-1 increases basal prostacyclin release and the expression of surface procoagulant activity by endothelial cells [12–14,46,47]. It is conceivable that increases in secretory activity such as these, several requiring gene transcription or protein synthesis, might result in enhanced polyamine turnover. To address this point, cultures of endothelial cells were incubated with these agonists for times and in concentrations equal to or greater than those previously shown to have secretagogue activity, but such treatment did not affect the subsequent uptake of polyamines by these cells.

It is difficult to assess the importance *in vivo* of endothelial polyamine uptake under normal circumstances, although there is preliminary evidence that, in addition to their growth-regulatory functions, the polyamines may play a role in signal transduction by modulating intracellular free Ca^{2+} levels [48]. Malignancy, however, frequently results in elevation of plasma polyamine levels and, since uptake is markedly enhanced when intracellular polyamine levels are depleted, this could be a complicating factor in the use of inhibitors of polyamine biosynthesis in cancer chemotherapy. In particular, the accumulation by vascular endothelial cells of polyamines and, more importantly, polyamine analogues, could have pharmacological significance. The ability

to manipulate the polyamine-transport systems of the vascular endothelium selectivity could also provide a novel therapeutic tool for the regulation of angiogenesis.

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